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PROSTATE CANCER

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14. ABSTRACT An important mechanism mediating androgen receptor (AR) reactivation in prostate cancers (PCa) that relapse after androgen deprivation therapy (castration resistant prostate cancer, CRPC) is increased intratumoral conversion of weak adrenal androgens into testosterone and DHT. We show that AR activity in castration resistant VCaP PCa xenografts is restored through CYP17A1 dependent de novo androgen synthesis, and that abiraterone treatment selects for xenografts with markedly increased intratumoral CYP17A1 expression. Consistent with these results, intratumoral expression of CYP17A1 is markedly increased in tumor biopsies from CRPC patients after CYP17A1 inhibitor therapy. We further show that CRPC cells expressing a progesterone responsive T877Amutant AR are not CYP17A1 dependent, but that AR activity in these cells is still steroid dependent and mediated by upstream CYP11A1 dependent intratumoral pregnenolone/progesterone synthesis. These findings indicate that CYP17A1 inhibitor resistant tumors may still be steroid dependent and respond to therapies that further suppress de novo intratumoral steroid synthesis.					
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INTRODUCTION

The majority of prostate cancers (PCa) are androgen dependent, and androgen deprivation therapy (ADT) remains as the standard treatment for non-organ confined disease. Unfortunately, patients treated with ADT invariably relapse with rapidly progressive systemic PCa, which has been termed hormone refractory, androgen independent, or castration resistant prostate cancer (CRPC). Significantly, the androgen receptor (AR) is highly expressed in most cases of CRPC and appears to be transcriptionally active, but the molecular events mediating the progression to CRPC and apparent reactivation of AR transcriptional activity remain to be defined. Our previous data indicate that increased intratumoral production of testosterone from precursors (adrenal androgens and possibly endogenous sterols) contributes to the reactivation of AR transcriptional activity in CRPC. We proposed that tumors adapt to androgen deprivation therapy by increasing their synthesis of potent androgens from available weak adrenal androgens (and possibly from endogenous precursors), and that AKR1C3 is a key enzyme in this process. Our objectives were to test these hypotheses using cell line and xenograft models (Aim 1) and by measuring androgen and androgen metabolite levels in patients who progress to CRPC (Aim 2).

BODY

Over the past several years, work accomplished by others and us has led to the general acceptance that intratumoral androgen synthesis contributes to CRPC. Indeed, the first new drug targeting residual androgen synthesis in CRPC, abiraterone, was very recently approved by the FDA. However, despite drugs such as abiraterone, an inhibitor of the enzyme CYP17A1 that is required for synthesis of DHEA (an androgen precursor), patients still eventually relapse. Therefore, our studies have been extended to now encompass efforts to understand the basis for progression after treatment with CYP17A1 inhibitors.

For this final report, we are attaching the published manuscripts below that cover material outlined in previous annual reports.

Taplin,M.E., Manola,J., Oh,W.K., Kantoff,P.W., Bubley,G.J., Smith,M., Barb,D., Mantzoros,C., Gelmann,E.P., and Balk,S.P. (2008). A phase II study of mifepristone (RU-486) in castration-resistant prostate cancer, with a correlative assessment of androgen-related hormones. *BJU. Int.* 101, 1084-1089.

Cai,C., Wang,H., Xu,Y., Chen,S., and Balk,S.P. (2009). Reactivation of Androgen Receptor-Regulated TMPRSS2:ERG Gene Expression in Castration-Resistant Prostate Cancer. *Cancer Res.* 69, 6027-6032.

Taplin,M-E., Regan, M.M., Ko,Y-J., Bubley,G.J., Duggan,S.E., Werner,L., Beer, T.M., Ryan,C.W., Mathew,P., Tu,S-M., Denmeade,S.R., Oh,W.K., Sartor,O., Mantzoros,C.S., Rittmaster,R., Kantoff,P.W., and Balk,S.P. (2009). Phase II study of androgen synthesis inhibition with ketoconazole, hydrocortisone and dutasteride (KHAD) in asymptomatic castration resistant prostate cancer. *Clin. Cancer Res.*15, 7099-7105.

In addition, we summarize data below that are currently under review for publication.

VCaP PCa cells have substantial basal AR transcriptional activity.

VCaP cells, derived from a vertebral metastasis in a patient with CRPC, express wild-type AR and AR-regulated genes such as PSA and the TMPRSS2:ERG fusion gene. Although androgen responsive, VCaP cells, like C4-2 cells (derived from a LNCaP xenograft that relapsed after castration), express substantial basal PSA when cultured in steroid depleted serum (Figure 1A). VCaP also had substantial nuclear AR under these conditions, consistent with the basal PSA expression, which was increased by DHT (Figure 1B). ChIP further showed that substantial AR in VCaP cells, but not LNCaP cells, was bound to the androgen responsive element (ARE) in the PSA gene enhancer in the absence of exogenous androgens (Figure 1C).

To determine whether de novo steroid synthesis may contribute to basal AR activity in VCaP or C4-2 cells, we next accessed the expression of steroid biosynthetic enzymes (Figure 1D). Transcripts encoding each enzyme were readily detected and moderately higher in C4-2 versus LNCaP, while VCaP had markedly higher expression of AKR1C3 and HSD17B6, but lower CYP11A1 compared to C4-2 and LNCaP cells (Figure 1E). Together these findings suggested that basal AR activity in VCaP and C4-2 cells may be mediated by CYP17A1 dependent de novo androgen synthesis.

Basal AR activity in VCaP cells and relapsed xenografts is CYP17A1 dependent.

Significantly, a CYP17A1 inhibitor (ketoconazole) decreased basal PSA expression in VCaP cells, but not C4-2 cells (Figure 2A). This block could be overcome by a downstream androgen precursor, androstenedione, indicating that it was due to CYP17A1 blockade and not a toxic effect (Figure 2B). Abiraterone, a more specific CYP17A1 inhibitor, similarly decreased basal PSA and ERG (from the AR regulated TMPRSS2:ERG fusion gene) expression in VCaP cells, but not in C4-2 cells, further indicating that basal AR activity in VCaP, but not C4-2, was CYP17A1 dependent (Figure 2C). AR protein in VCaP cells was also decreased by abiraterone, consistent with the decreased stability of the unliganded AR. In contrast, PSA expression stimulated by androstenedione (downstream of CYP17A1) was not blocked by abiraterone (Figure 2D). RT-PCR further established that abiraterone was decreasing expression of AR-regulated transcripts encoding PSA, TMPRSS2, ERG, PLZF, and FKBP5, and that this block could be bypassed by androstenedione (Figure 2E).

Rodent adrenal glands express low or undetectable levels of CYP17, so serum levels of testosterone and androgen precursors are extremely low in castrated male and in female mice. In a previous study we showed that VCaP xenografts that relapsed after castration had restored AR transcriptional activity and had increased expression of androgen synthetic genes including AKR1C3, but CYP17A1 was not consistently increased. To assess the effects of inhibiting CYP17A1 in vivo, castration resistant VCaP xenografts were transplanted into female mice. Established tumors in each mouse were biopsied, and the mice were then treated with 0.5 mg abiraterone ip every other day for 8 days followed by repeat biopsies. Comparison of mRNA from pre- and post-treatment biopsies showed that abiraterone decreased the expression of multiple androgen-regulated genes (PSA, TMPRSS2, and ERG), but did not decrease AR mRNA, consistent with decreased AR transcriptional activity (Figure 2F).

Immunohistochemistry also indicated decreased PSA protein and decreased nuclear ERG and AR after abiraterone treatment (Figure 2G). Moreover, Ki67-positive cells were greatly decreased in the abiraterone treated tumors, indicating that tumor growth was suppressed (Figure 2G). Measurement of androgen levels in tumor biopsies confirmed that testosterone and DHT were decreased by abiraterone (Figure 2H).

Basal AR activity in VCaP cells and castration resistant VCaP xenografts is AKR1C3 dependent.

Previously we showed that AKR1C3, the prostatic enzyme mediating reduction of androstenedione to testosterone, was increased in human CRPC biopsies relative to primary PCa, and that its expression was increased in VCaP xenografts that relapse after castration. AKR1C3 mRNA was also much higher in VCaP than LNCaP or C4-2 cells (Figure 1E), while at the protein level AKR1C3 was only detected in VCaP cells (Figure 3A). As specific AKR1C3 inhibitors are not yet available, we generated stable AKR1C3 knock-down cells by infecting VCaP cells with an AKR1C3 shRNA lentivirus. PSA in the absence of DHT was dramatically decreased in VCaP-shAKR1C3 cells, and could be restored by DHT (Figure 3B). In contrast, androstenedione, a very weak direct AR agonist, only minimally increased PSA in the VCaP-shAKR1C3 cells (Figure 3C), confirming that AKR1C3 was mediating the synthesis of physiologically significant levels of testosterone from androstenedione. Although not selective, the nonsteroidal anti-inflammatory drug indomethacin at micromolar concentrations can also inhibit AKR1C3. Similarly to the effect of shAKR1C3, indomethacin decreased basal and androstenedione stimulated PSA expression in VCaP cells, but not in C4-2 cells (Figure 3D). RT-PCR confirmed that indomethacin was decreasing mRNA levels of PSA, TMPRSS2, and PLZF, and that this effect could be bypassed by DHT (Figure 3E).

To examine the effects of inhibiting AKR1C3 *in vivo* in castration resistant VCaP xenografts, we castrated another series of male mice bearing VCaP xenografts. To inhibit AKR1C3, we treated these castrated male mice bearing relapsed VCaP xenografts for 2 weeks with indomethacin, which resulted in decreased PSA, ERG, and TMPRSS2 mRNA expression (Figure 3F). AR mRNA was slightly decreased, although this was not significant. Immunohistochemistry also indicated decreased PSA and ERG protein, decreased nuclear expression of ERG and AR, and a decrease in Ki-67-positive cells in response to indomethacin (Figure 3G). Finally, consistent with AKR1C3 inhibition, testosterone and DHT levels were decreased in the treated tumors (Figure 3H).

AR reactivation in castration resistant VCaP cell line is dependent on de novo androgen synthesis.

To address whether the effects of inhibitors *in vivo* were due to blocking low levels of CYP17A1 or AKR1C3 in the adrenals or other tissues, rather than suppressing *de novo* synthesis in the xenografts, we used a relapsed VCaP xenograft to generate a castration resistant VCaP cell line (VCS2). Significantly, AR transcriptional activity in steroid-depleted medium, as assessed by PSA expression, was markedly increased in these VCS2 cells compared to the parental VCaP cells and was only weakly further induced by DHT (Figure 4A). Consistent with this increased basal activity, expression of AR and androgen synthetic enzymes were increased in the VCS2 cells (Figure 4A and 4B). PSA expression in the VCS2 cells could be markedly decreased by indomethacin (Figure 4C) and by AKR1C3 siRNA (Figure 4D), confirming that the high level basal PSA expression was AKR1C3 dependent. Basal PSA expression was also markedly suppressed by abiraterone (Figure 4E), and could be even further suppressed by combined abiraterone and indomethacin treatment (Figure 4F), confirming that reactivated AR activity in these castration resistant tumors is dependent on enhanced CYP17A1 and AKR1C3 dependent intratumoral *de novo* androgen synthesis.

Intratumoral CYP11A1 dependent pregnenolone/progesterone synthesis drives CYP17A1 independent AR activity in cells expressing progesterone responsive T877A mutant AR.

In contrast to VCaP cells, C4-2 cells express very low levels of AKR1C3 and their basal AR activity was not blocked by CYP17A1 inhibitors (see Figures 1-3). Significantly, LNCaP and C4-2 cells express a mutant AR (T877A) that can be stimulated by certain AR antagonists and steroids including progesterone that do not substantially activate wildtype AR (Figure 5A) (28, 29). Indeed, both DHT and progesterone can strongly stimulate endogenous AR activity in LNCaP cells, and are more potent than androstenedione or testosterone (Figure 5B). These observations suggested that basal AR activity in C4-2 cells may be driven by progesterone, which is synthesized from cholesterol upstream of CYP17A1 by the sequential actions of CYP11A1 and HSD3B1 or HSD3B2 (see Figure 1D). To test this hypothesis, we first examined high passage number LNCaP cells (LN-HP cells) that express substantial PSA in steroid depleted medium. As shown in figure 5C, stable infection with a CYP11A1 shRNA lentivirus markedly decreased basal PSA mRNA, and this was prevented by DHT or progesterone. Interestingly, although CYP11A1 has not been characterized as an AR regulated gene, its expression also was increased by progesterone and DHT. Immunoblotting confirmed the marked decrease in basal PSA in response to CYP11A1 shRNA, which could be restored with DHT (Figure 5D). In C4-2 cells, basal levels of AR regulated transcripts (PSA, PLZF, and FKBP5) and PSA protein expression were similarly decreased by CYP11A1 shRNA, and could be restored by DHT or progesterone (Figure 5E and F). Finally, CYP11A1 shRNA restored androgen stimulated growth of C4-2 in steroid depleted medium (Figure 5G). Together these findings indicate that CYP11A1 dependent intratumoral synthesis of progesterone is driving the high levels of AR activity in these cells with a T877A mutant AR.

Increased CYP17A1 dependent intratumoral de novo androgen synthesis mediates resistance to CYP17A1 inhibitors.

To gain insight into possible mechanisms of acquired resistance to CYP17A1 inhibition, we treated a series of mice bearing castration resistant VCaP xenografts with abiraterone. All mice responded initially (see Figure 2), and there was no substantial or consistent change in CYP17A1 expression in the responding xenografts from mice sacrificed after 8 days of treatment (Figure 6A). We then biopsied another group of castration resistant VCaP xenografts and then treated with abiraterone until relapse based on renewed rapid growth (~4 weeks). PSA expression, which declined initially in response to abiraterone, returned to levels that were close to or above baseline in 5 of 6 xenografts, indicating restored AR activity (Figure 6B). Changes in the expression of AKR1C3 and AR were modest and variable (Figure 6C), and no AR ligand binding domain mutations were detected by cDNA sequencing (not shown). In contrast, CYP17A1 expression was increased in all relapsed xenografts, with increases of ~100-fold in 2 of the xenografts. This selection for tumors cells with elevated CYP17A1 in response to abiraterone treatment indicates that upregulation of CYP17A1 provides a growth advantage and is a mechanism that can contribute to the development of abiraterone resistance.

We reported previously that mRNA for enzymes mediating conversion of weak adrenal androgens (DHEA and androstenedione) to testosterone and DHT (including HSD17B2, AKR1C3, and SRD5A1) were increased in metastatic CRPC clinical samples. There was also an increase in median CYP11A1, but CYP17A1 was not increased, suggesting that these tumors may not carry out significant de novo androgen synthesis or that CYP17A1 is not rate limiting (Figure 6C). To address these alternatives, we used quantitative real time RT-PCR to directly

compare expression of CYP17A1 in CRPC clinical samples versus castration resistant VCaP xenografts. Significantly, median CYP17A1 levels in the CRPC clinical samples and castration resistant VCaP xenografts (VCaP-CR) were comparable, while median CYP11A1 was higher in the CRPC samples, indicating that many or most of the clinical samples expressed these enzymes at levels that were consistent with physiologically significant levels of androgen synthesis and AR reactivation (Figure 6D).

While our data indicate that CYP17A1 mRNA is not consistently increased in CRPC, this may reflect the high levels of circulating CYP17A1 dependent weak androgens produced by the adrenal glands. Therefore, to determine whether suppression of adrenal gland steroid synthesis in CRPC patients through treatment with a CYP17A1 inhibitor selects for tumor cells with increased CYP17A1, we next examined tumor samples obtained from bone marrow biopsies in CRPC patients treated with high dose ketoconazole (CYP17A1 inhibitor) and dutasteride (5 α -reductase inhibitor). Biopsies from four patients obtained after one month of treatment (P1-P4), when serum PSA levels were declining, and from two patients who were relapsing after responding to ketoconazole for > 1 year, all showed levels of CYP17A1 mRNA that were markedly higher than the median expression in biopsies from CRPC patients who were not being treated with a CYP17A1 inhibitor (Figure 6D) (each circle reflects the level measured in an independent biopsy taken from the same region). In contrast, there were no apparent further increases in AKR1C3 or CYP11A1 expression.

Figure Legends

Figure 1. VCaP cells have substantial basal AR activity. A, VCaP, LNCaP, or C4-2 cells grown in medium with 5% charcoal-dextran stripped fetal bovine serum (CSS) were treated with 0, 0.1, 1, or 10 nM DHT for 24h and then immunoblotted for PSA and β -actin (loading control). B, VCaP or LNCaP cells in CSS medium were treated with 10 nM DHT for 0, 0.5 or 4h and then fractionated into nuclear extracts (NE) or cytoplasmic extracts (CE). NE, CE or whole cell lysates were immunoblotted for AR, β -tubulin (cytoplasm control), or β -actin (loading control). C, VCaP or LNCaP cells in CSS medium were treated with 0, 1, or 10 nM DHT for 4h and then subjected to ChIP assay. The DNA fragments were PCR amplified and normalized to input to measure binding to the PSA enhancer ARE or irrelevant site (negative control). D, androgen synthesis pathway. E, LNCaP, C4-2, or VCaP cells were subjected to quantitative real time RT-PCR (qRT-PCR) to measure CYP17A1, AKR1C3, HSD3B2, CYP11A1, SRD5A1, HSD17B3, or HSD17B6 (major enzyme mediating the regeneration of DHT from its reduced metabolite). Equal amounts of cellular RNA were coamplified with 18S RNA as an internal control.

Figure 2. Basal AR activity in VCaP is dependent on CYP17A1. A, VCaP, LNCaP, or C4-2 cells in CSS medium were treated with 0, 2, or 5 μ M ketoconazole (Keto) for 24h and then immunoblotted. B, VCaP cells in CSS medium were treated with 0, 1, 10, or 100 nM androstenedione (Ad) with or without 2 μ M ketoconazole for 24h. C, VCaP or C4-2 cells in CSS medium were treated with 0, 2, or 5 μ M abiraterone (Abir) for 24h. D, VCaP cells in CSS medium were treated with 0 or 100 nM androstenedione with or without 2 μ M abiraterone and then immunoblotted or (E) subjected to qRT-PCR. F, tissue samples of recurrent VCaP xenografts were taken from each mouse pre-treatment and post-treatment with abiraterone (0.5 mg/2d for 8 days) and then subjected to RT-PCR (n=4) or (G) immunohistochemistry. H, testosterone or DHT levels in recurrent VCaP xenograft tumors were measured pre- or post-

abiraterone treatment. DHEA and androstenedione levels were below the level of detection pre- and post-treatment.

Figure 3. Basal AR activity in VCaP is AKR1C3 dependent. A, LNCaP, C4-2, or VCaP cells were immunoblotted for AKR1C3 or β -tubulin (loading control). B and C, VCaP cells stably infected with lentivirus expressing either GFP shRNA or AKR1C3 shRNA (Open Biosystems) in CSS medium were treated with 10 nM DHT or 100 nM androstenedione (Ad) for 24h, and then immunoblotted. D, VCaP or C4-2 cells in CSS medium were treated with 0, 20, or 40 μ M indomethacin for 24h. E, VCaP cells in CSS medium were treated with 0, 20, or 40 μ M indomethacin with or without 10nM DHT and subjected to qRT-PCR. F, mice bearing relapsed VCaP xenografts were treated with indomethacin for 2 weeks (~0.25 mg per day in drinking water) and tissue samples taken pre- and post-therapy from tumors (n=5) were analyzed by RT-PCR as indicated or (G) by immunohistochemistry for PSA, ERG, AR, and Ki67. H, testosterone and DHT levels in recurrent xenograft tumors in transplanted female scid mice (n=3) were measured pre- or post-indomethacin (Indo) treatment.

Figure 4. Inhibition of CYP17A1 and AKR1C3 suppresses AR activity in a castration-resistant VCaP cell line. A, the VCS2 cells generated from a relapsed VCaP xenograft were passaged in culture in 8%CSS/2%FBS medium. They were switched to CSS medium for 3 days, then treated for 1 day with 0, 1, or 10 nM DHT, and proteins were then immunoblotted. Long (L) and short (S) exposures are shown for PSA. B, VCaP or VCS2 cells were subjected to qRT-PCR to measure CYP17A1, AKR1C3, or HSD3B2. C, VCS2 cells in CSS medium were treated for 24h with 0, 20, or 40 μ M indomethacin and then immunoblotted. D, VCS2 cells in CSS medium were transfected with 20 nM AKR1C3 siRNA (Dharmacon) for 2d and then treated for 24h with vehicle (ethanol) or 10 nM DHT and immunoblotted for PSA and AKR1C3. E, VCS2 cells in CSS medium were treated for 24h with 0, 2, or 5 μ M abiraterone and then immunoblotted. F, VCS2 cells in CSS medium were treated for 24h with abiraterone (2 μ M) and indomethacin (20 or 40 μ M) as indicated and then immunoblotted (note longer exposure compared to E).

Figure 5. Basal activity of T877A mutant AR in LNCaP and C4-2 is dependent on CYP11A1. A, COS-7 cells in 5% CSS medium were transfected with an androgen responsive element regulated luciferase reporter (ARE4-Luc) and wild-type AR or T877A mutant AR. Cells were then treated with vehicle (ethanol), DHT, testosterone (T), androstenedione (Ad), pregnenolone (Pn), progesterone (Pg), or 17 α -OH-progesterone (17 α -OH-Pg) (10 nM for each treatment). Reporter activity was normalized to the cotransfected CMV-Renilla-Luc. B, LNCaP cells in 5% CSS medium were treated with vehicle (-), with 0.1, 1, or 10 nM DHT, progesterone, or testosterone, or with 1 or 10 nM androstenedione and then immunoblotted. C, high-passage number LNCaP cell line (>50, LN-HP) was stably infected with lentivirus expressing either GFP shRNA or CYP11A1 shRNA (Open Biosystems), then treated with vehicle (ethanol), 10 nM DHT, or 10 nM progesterone, and analyzed by qRT-PCR for PSA and CYP11A1. D, LN-HP cells infected with GFP or AKR1C3 shRNA lentivirus were treated with 0, 1, 10 nM DHT and immunoblotted. E, C4-2 cells stably infected with lentivirus expressing either GFP shRNA or CYP11A1 shRNA were treated with 0, 1, 10 nM DHT and analyzed by RT-PCR or (F) treated with 0, 1, 10 nM progesterone and immunoblotted. G, numbers of C4-2-shGFP or C4-2-shCYP11A1 cells cultured in 5% CSS medium and treated with or without DHT were measured using MTT assay after 7 days.

Figure 6. CYP17A1 inhibition in CRPC selects for increased CYP17A1. A, mice bearing recurrent VCaP xenografts were treated with abiraterone for a short period (n=4, 0.5 mg/d for 8 days by i.p. injection) or (B) for extended periods until relapse (n=6, 0.5 mg/ml in drinking water for 4-6 weeks). RNA extracted from tumor biopsies pre- or post-treatment was analyzed by qRT-PCR with GAPDH coamplified as an internal control. The change of gene expression was presented as Log2(fold change). C, Affymetrix microarray expression data for CYP17A1 and CYP11A1 in 27 primary tumors (no hormonal therapy) and 29 CRPC bone marrow metastases. D, expression of CYP17A1, AKR1C3, and CYP11A1 were assessed by qRT-PCR in 29 CRPC bone marrow biopsy tumor samples, 3 relapsed castration resistant VCaP xenografts (VCaP-CR), 3 relapsed castration resistant LNCaP xenografts (LN-CR), 2-3 bone marrow biopsy tumor samples each from 6 ketoconazole-treated patient (P1-P6), and 4 bone marrow biopsy samples from CRPC patients that contained only normal bone marrow (NBM), with GAPDH amplified as an internal control.

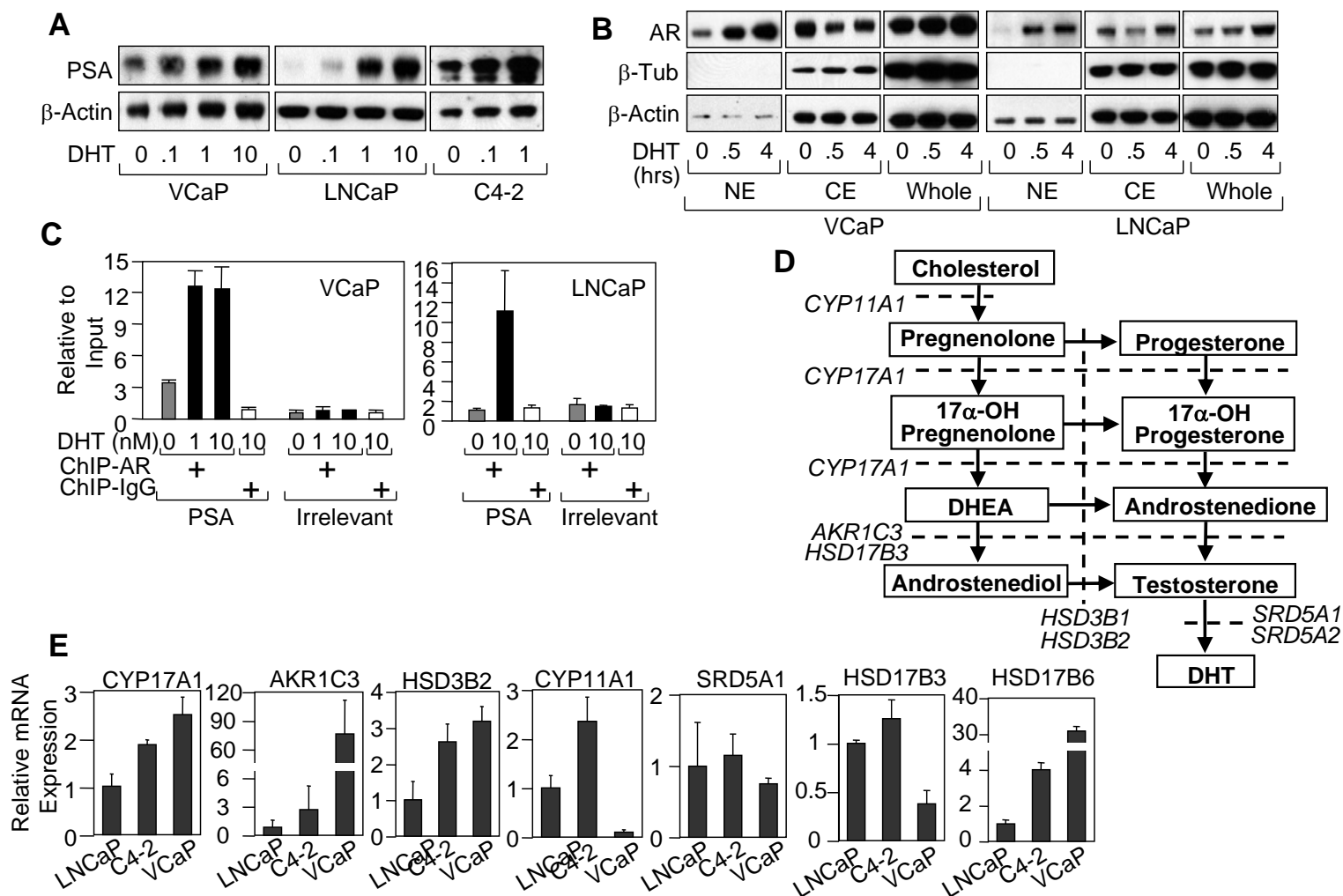


Figure 1

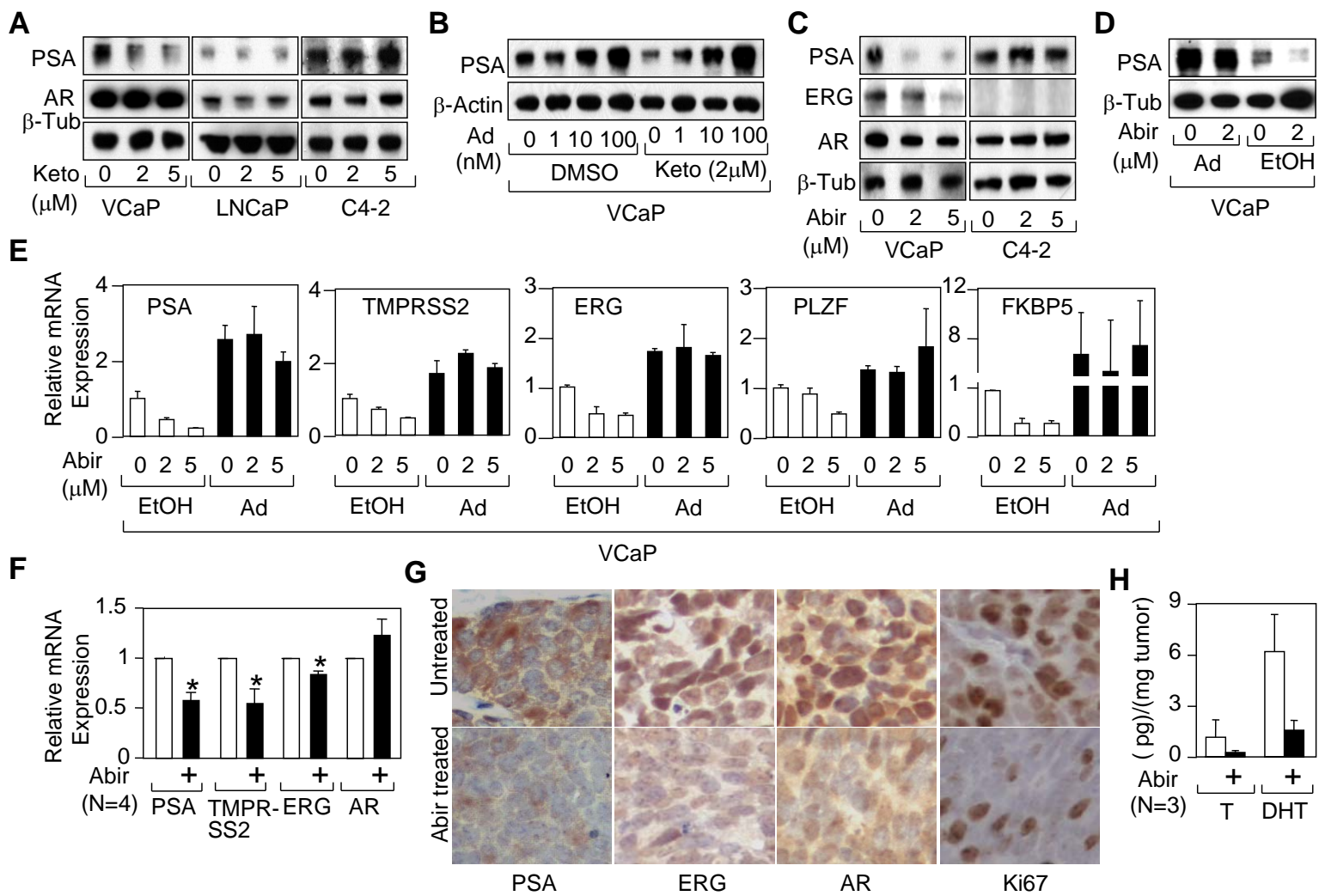


Figure 2

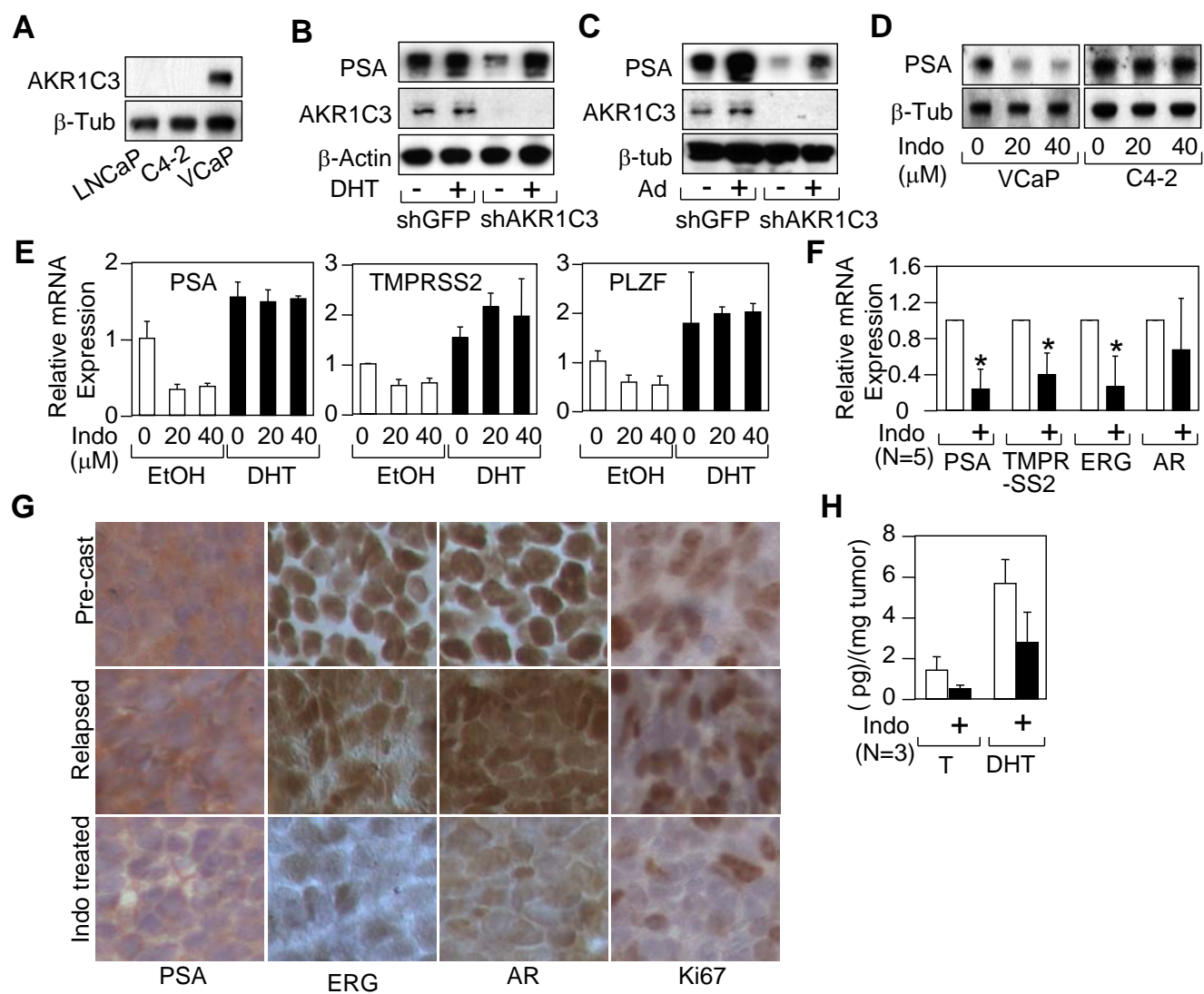


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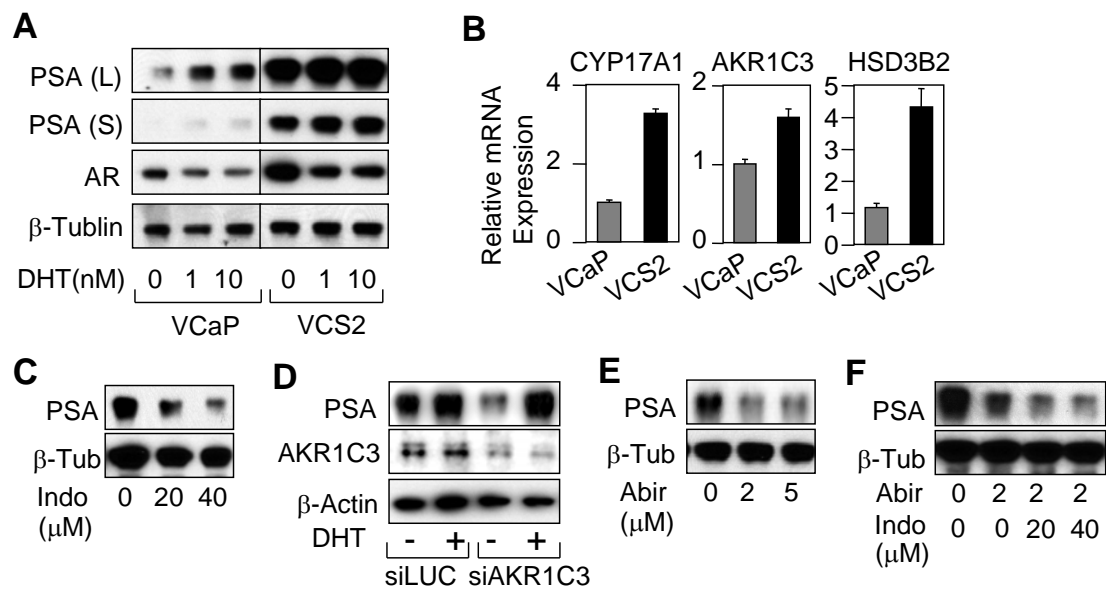


Figure 4

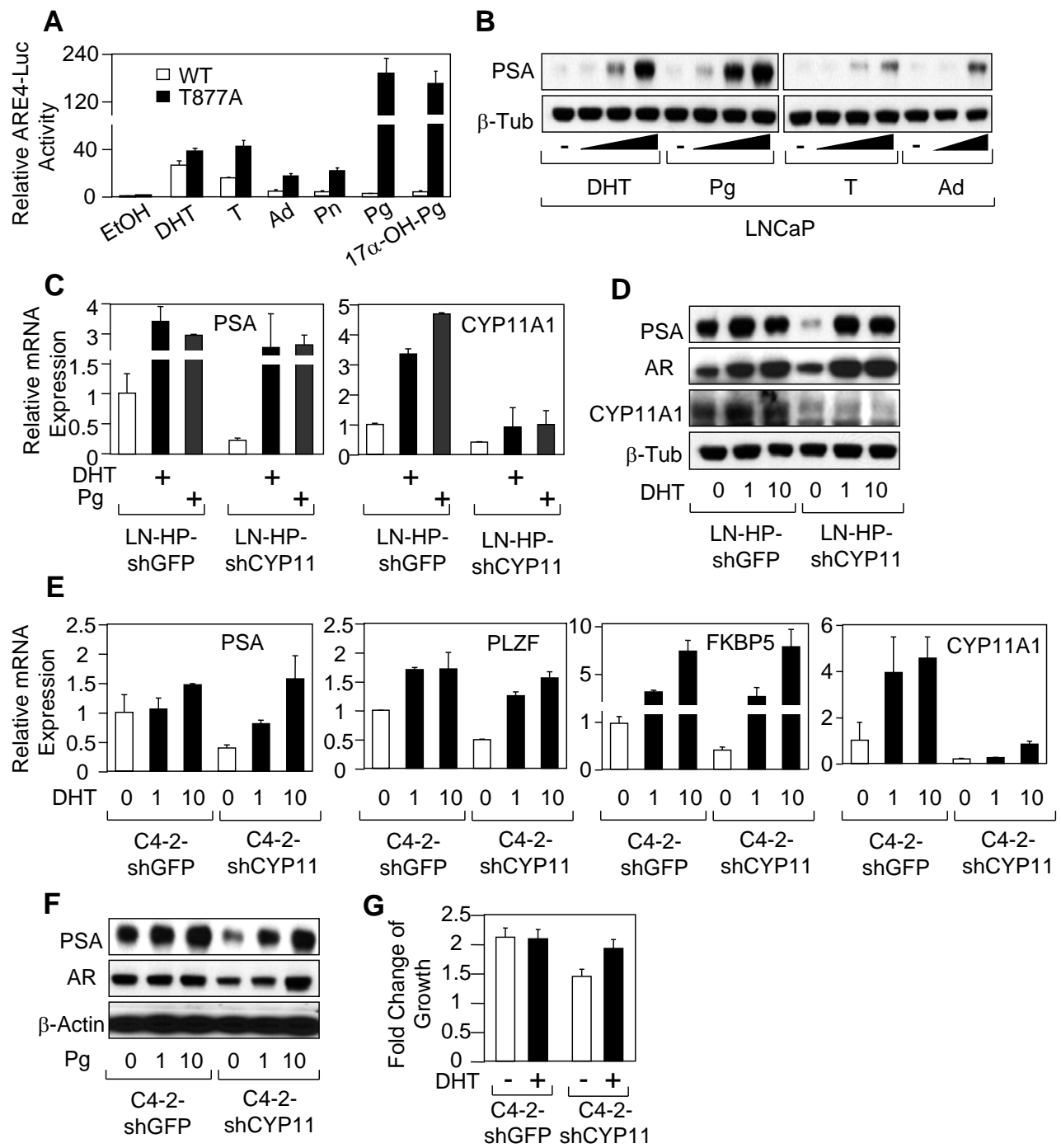


Figure 5

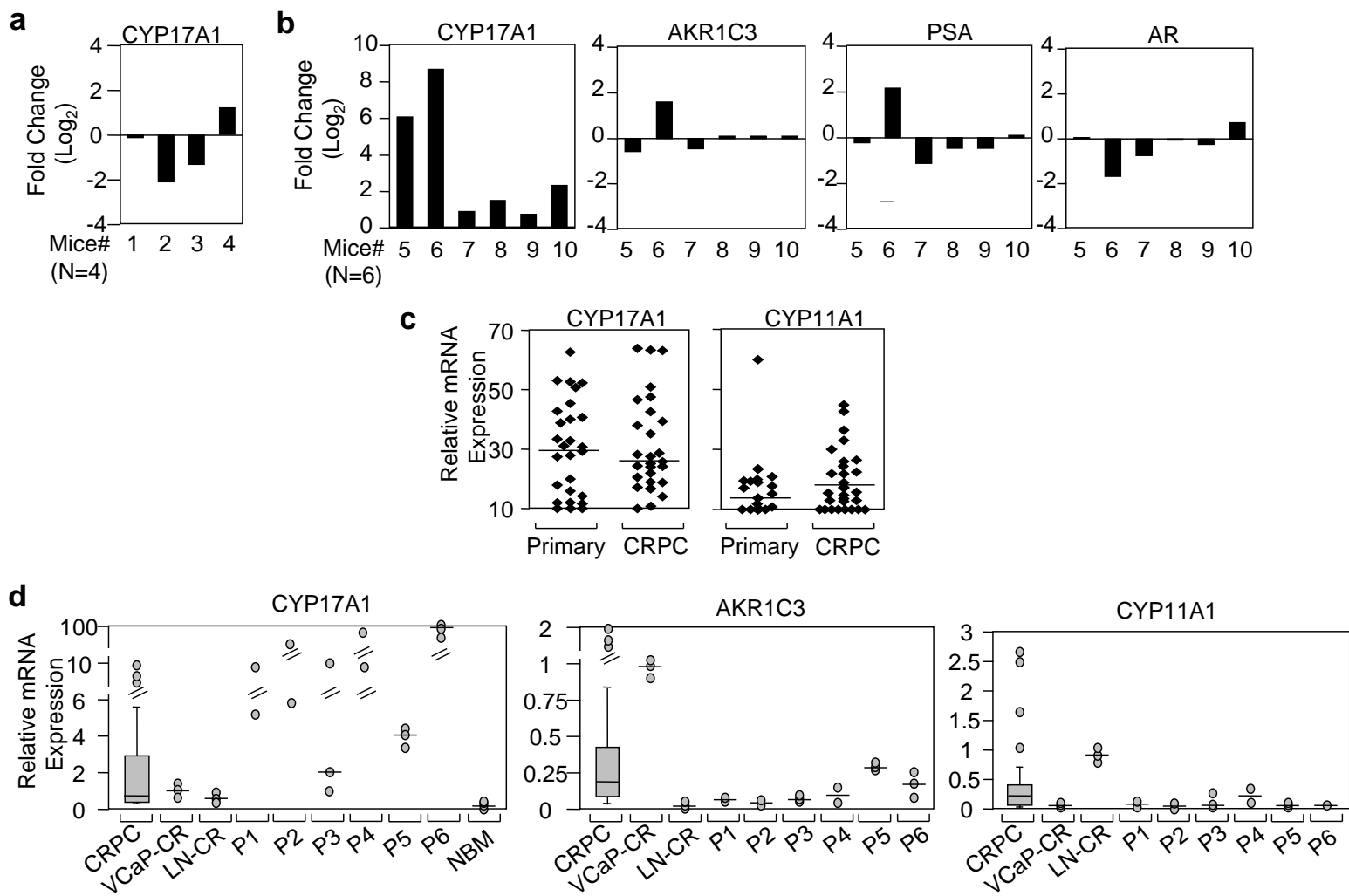


Figure 6

KEY RESEARCH ACCOMPLISHMENTS

- Established VCaP as a xenograft model for increased androgen synthesis in prostate cancer progression
- Demonstrated efficacy of an AKR1C3 inhibitor in this model
- Expanded collection of serum samples for hormone measurements
- Assessed hormone levels in a clinical trial of androgen synthesis inhibitors in CRPC

REPORTABLE OUTCOMES

Three publications and additional data outlined above that is currently submitted for publication

PERSONNEL

The following BIDMC personnel received pay from this research effort:

Steven Balk – Principal Investigator

Stephen Duggan – Technician

Fang Zi – Post Doctoral Fellow

Sarah Gulla – Research Assistant

Nicholas Simon – Research Assistant

CONCLUSIONS

An important mechanism mediating androgen receptor (AR) reactivation in prostate cancers (PCa) that relapse after androgen deprivation therapy (castration resistant prostate cancer, CRPC) is increased intratumoral conversion of weak adrenal androgens, including dehydroepiandrosterone (DHEA) and androstenedione, into testosterone and dihydrotestosterone (DHT). DHEA and androstenedione are synthesized by the adrenals through the sequential actions of CYP11A1 and CYP17A1, so that CYP17A1 inhibitors such as abiraterone are effective therapies for CRPC. However, the significance of intratumoral CYP17A1 and de novo androgen synthesis in CRPC, and the mechanisms contributing to CYP17A1 inhibitor resistance/relapse, remain to be determined. We show that AR activity in castration resistant VCaP PCa xenografts is restored through CYP17A1 dependent de novo androgen synthesis, and that abiraterone treatment selects for xenografts with markedly increased intratumoral CYP17A1 expression. Consistent with these results, intratumoral expression of CYP17A1 is markedly increased in tumor biopsies from CRPC patients after CYP17A1 inhibitor therapy. We further show that CRPC cells expressing a progesterone responsive T877A mutant AR are not CYP17A1 dependent, but that AR activity in these cells is still steroid dependent and mediated by upstream CYP11A1 dependent intratumoral pregnenolone/progesterone synthesis. These findings indicate that CYP17A1 inhibitor resistant tumors may still be steroid dependent and respond to therapies that further suppress de novo intratumoral steroid synthesis.

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Cai,C., Wang,H., Xu,Y., Chen,S., and Balk,S.P. (2009). Reactivation of Androgen Receptor-Regulated TMPRSS2:ERG Gene Expression in Castration-Resistant Prostate Cancer. *Cancer Res.* 69, 6027-6032.

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APPENDICES

Three publications

Cancer Research

Reactivation of Androgen Receptor–Regulated *TMPRSS2:ERG* Gene Expression in Castration-Resistant Prostate Cancer

Changmeng Cai, Hongyun Wang, Youyuan Xu, et al.

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Reactivation of Androgen Receptor–Regulated *TMPRSS2:ERG* Gene Expression in Castration-Resistant Prostate Cancer

Changmeng Cai, Hongyun Wang, Youyuan Xu, Shaoyong Chen, and Steven P. Balk

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Abstract

It seems clear that androgen receptor (AR)–regulated expression of the *TMPRSS2:ERG* fusion gene plays an early role in prostate cancer (PC) development or progression, but the extent to which *TMPRSS2:ERG* is down-regulated in response to androgen deprivation therapy (ADT) and whether AR reactivates *TMPRSS2:ERG* expression in castration-resistant PC (CRPC) have not been determined. We show that ERG message levels in *TMPRSS2:ERG* fusion-positive CRPC are comparable with the levels in fusion gene–positive primary PC, consistent with the conclusion that the *TMPRSS2:ERG* expression is reactivated by AR in CRPC. To further assess whether *TMPRSS2:ERG* expression is initially down-regulated in response to ADT, we examined VCaP cells, which express the *TMPRSS2:ERG* fusion gene, and xenografts. ERG message and protein rapidly declined in response to removal of androgen *in vitro* and castration *in vivo*. Moreover, as observed in the clinical samples, ERG expression was fully restored in the VCaP xenografts that relapsed after castration, coincident with AR reactivation. AR reactivation in the relapsed xenografts was also associated with marked increases in mRNA encoding AR and androgen synthetic enzymes. These results show that expression of *TMPRSS2:ERG*, similarly to other AR-regulated genes, is restored in CRPC and may contribute to tumor progression. [Cancer Res 2009;69(15):6027–32]

Introduction

A major breakthrough in prostate cancer (PC) was identification of recurrent fusions between androgen-regulated *TMPRSS2* and Ets transcription factor genes (primarily *ERG*), placing the *Ets* genes under androgen-stimulated regulation of *TMPRSS2* (1). Remarkably, this fusion is in preneoplastic lesions and ~50% of primary PC, consistent with an early role in tumor development (1–6). The standard treatment for locally recurrent/metastatic PC is androgen deprivation therapy (ADT), but patients invariably relapse with more aggressive tumors termed castration-resistant PC (CRPC). Significantly, androgen receptor (AR) is expressed at high levels in CRPC, as are multiple AR-regulated genes, indicating that AR transcriptional activity is at least partially reactivated (7, 8). Mechanisms contributing to this reactivation include increased intratumoral androgen accumulation/synthesis (8–12), AR over-

expression, AR mutations (in AR antagonist–treated patients), and activation of kinase pathways that enhance AR activity.

TMPRSS2 is decreased in response to ADT (13), and it is presumed that *TMPRSS2:ERG* expression would also be decreased, which may contribute to responses, but this has not been shown directly in patients. The extent to which the *TMPRSS2:ERG* gene is expressed in CRPC and contributes to relapse is also unclear. One study of CRPC with the *TMPRSS2:ERG* gene found that it was not expressed, but this was in atypical AR-negative tumors (6). In contrast, the initial identification of fusion gene transcripts included CRPC tumors, although these were a small subset of outliers expressing very high ERG message levels (1). Therefore, to determine the extent to which *TMPRSS2:ERG* gene expression is reactivated in CRPC, we examined ERG expression in *TMPRSS2:ERG* fusion-positive primary androgen-dependent PC and CRPC clinical samples and in VCaP xenografts (14) before and after castration.

Materials and Methods

Cell culture and xenografts. Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). For DHT treatment, cells were first grown to 50% to 60% confluence in 5% charcoal/dextran-stripped FBS (CSS) medium for 3 d. VCaP xenografts were established in the flanks of male severe combined immunodeficient mice by injecting ~2 million cells in 50% Matrigel. When tumors reached ~1 cm, biopsies were obtained and the mice were castrated. Additional biopsies were obtained 4 d after castration, and the tumors were harvested at relapse. Frozen sections confirmed that samples contained predominantly nonnecrotic tumor.

Reverse transcription-PCR and immunoblotting. Real-time reverse transcription-PCR (RT-PCR) used 50 ng RNA, and the results were normalized by coamplification of 18S RNA (see Supplementary Data). Blots were incubated with anti-ERG (1:1,000, polyclonal; Santa Cruz Biotechnology), anti-prostate-specific antigen (PSA; 1:3,000, polyclonal; BioDesign), anti-AR (1:2,000, polyclonal; Upstate), anti-pAR(Ser⁸¹) (1:1,000, polyclonal; Upstate), or anti-actin (1:5,000, monoclonal; Abcam) and then with secondary antibodies (Promega).

Immunohistochemistry. Paraffin sections were boiled for 30 min in 10 mmol/L citrate buffer (pH 6.2) and blocked using 5% goat serum and avidin blocking solution (Vector). Primary antibodies, anti-AR (1:50) or anti-ERG (1:200), were added overnight at 4°C followed by biotinylated goat anti-rabbit antibody (1:400) and streptavidin-horseradish peroxidase (1:400; Vector). Slides were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin.

Results and Discussion

ERG is expressed at comparable levels in *TMPRSS2:ERG*-positive primary PC and CRPC. Using RT-PCR on RNA from previously described CRPC bone marrow metastases (8), we detected *TMPRSS2:ERG* transcripts (*TMPRSS2* exon 2–*ERG* exon 4) in 11 of 29 cases. Affymetrix oligonucleotide microarray data on these tumors versus a group of 27 microdissected primary PC (from the same study) were then examined for ERG expression.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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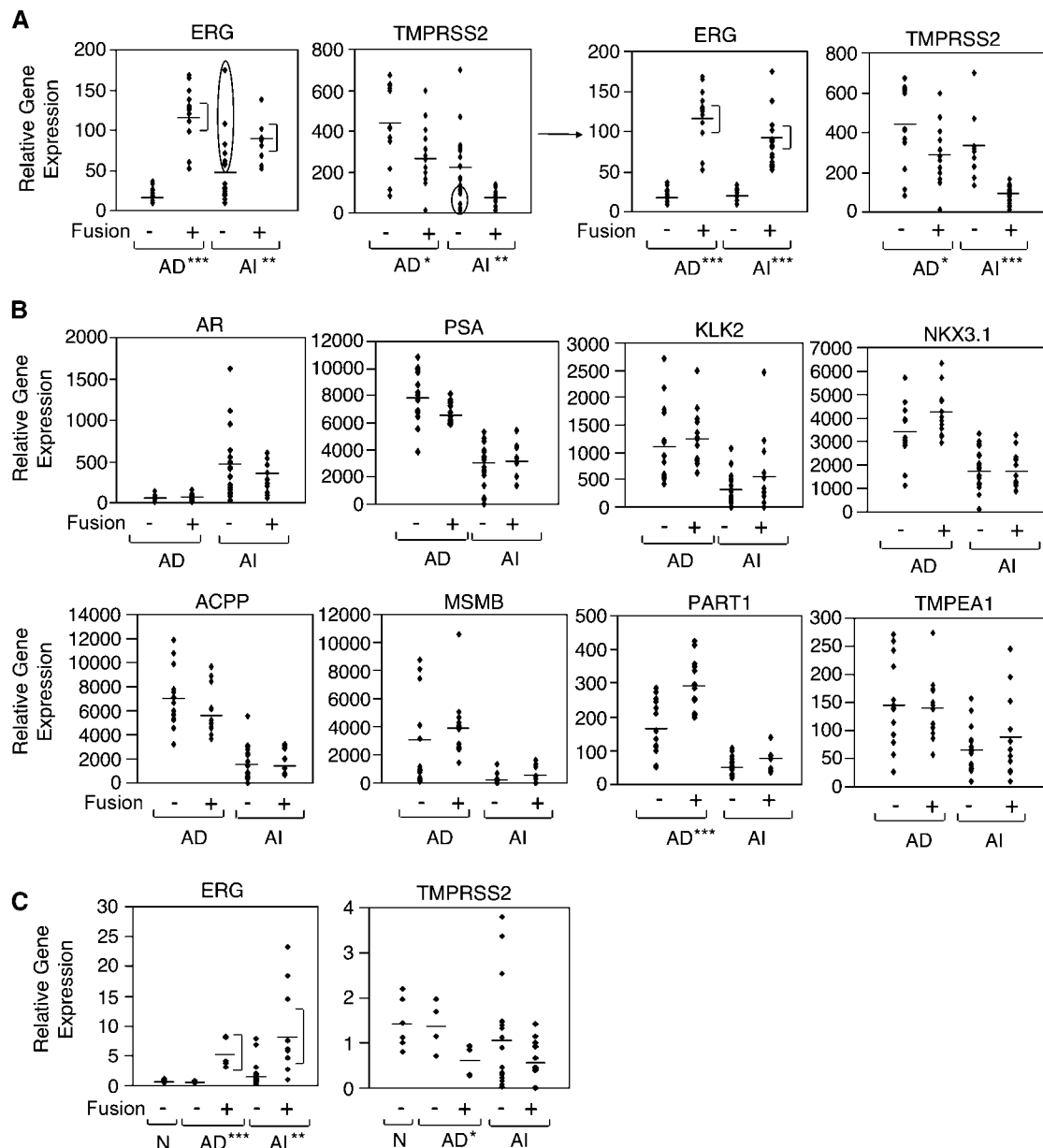


Figure 1. ERG expression in *TMPRSS2:ERG*-positive primary PC and CRPC. **A** and **B**, expression in fusion-negative versus fusion-positive primary PC [androgen-dependent (AD)] and CRPC [androgen-independent (AI)]. **C**, ERG and *TMPRSS2* expression by RT-PCR in these 29 CRPC tumors (AI) versus another group of 10 untreated primary PC (AD) and 6 normal prostates (N). Brackets, 95% confidence intervals for fusion-positive tumors. *P* values for differences between fusion-negative and fusion-positive androgen-dependent or androgen-independent tumors are shown. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Although the fusion status of the latter primary tumors was not known (RNA and tumor tissues were no longer available), ERG expression distinguished two nonoverlapping groups that presumably reflected fusion-negative (13 of 27) and fusion-positive tumors (14 of 27; Fig. 1A, AD, left).

The majority of the fusion-negative CRPC had low ERG levels that were comparable with the levels in the ERG low primary PC group (Fig. 1A, AI, left). However, ERG expression in six of the fusion-negative CRPC was higher (Fig. 1A, circled), suggesting *TMPRSS2:ERG* fusions that were not picked up by RT-PCR. These six samples also all had low *TMPRSS2* mRNA levels, suggesting a

TMPRSS2:ERG fusion and loss of *TMPRSS2* expression from one allele (Fig. 1A, left, circled). The panels on the right of Fig. 1A show these six tumors reclassified as fusion positive, which would indicate that ERG is expressed at comparable low levels in the fusion-negative primary (androgen dependent) and castration-resistant (androgen independent) tumors. Importantly, independent of how these six tumors are classified, ERG expression in the fusion-positive CRPC was significantly higher than in the fusion-negative CRPC. Moreover, ERG expression in the fusion-positive androgen-dependent and androgen-independent tumors was comparable based on overlapping confidence intervals, with a

>95% probability that levels in androgen independent tumors are at least 50% of those in androgen-dependent tumors (Fig. 1A).

We reported previously that AR mRNA was consistently increased in CRPC and that multiple AR-regulated genes were highly expressed (8). As shown in Fig. 1B, expression of AR-regulated and androgen-regulated genes was similarly increased in the fusion-positive versus fusion-negative CRPC, consistent with comparable AR reactivation in these tumors. Interestingly, whereas expression of most AR-regulated genes was 2- to 3-fold lower in CRPC, ERG expression seemed to be more fully restored, suggesting that factors in addition to AR may be further enhancing *TMPRSS2:ERG* expression in CRPC.

To confirm these results, we examined ERG expression by real-time RT-PCR in the CRPC samples and in another independent small set of fusion-negative and fusion-positive primary PC. Significantly, this analysis also showed that ERG expression in the fusion-positive CRPC samples was increased ~4-fold compared with the fusion-negative CRPC and was comparable with expression in the fusion-positive primary PC (Fig. 1C). Taken together, these results show that the *TMPRSS2:ERG* fusion gene is

expressed in CRPC at levels that are comparable with those in untreated primary PC, which presumably reflects at least in part reactivation of AR.

Expressions of *TMPRSS2:ERG* transcript and ERG protein are androgen stimulated in VCaP cells. Although the above data establish that *TMPRSS2:ERG* is comparably expressed in primary PC and CRPC, we have not yet been able to directly follow fusion gene expression *in vivo* in patients during ADT. Therefore, we next examined VCaP cells, which express AR and the common *TMPRSS2:ERG* fusion gene (14). Expression of an ~50 kDa protein, consistent with NH₂-terminal truncated ERG, could be induced rapidly and at low DHT levels (0.1 nmol/L) in VCaP but not fusion-negative LNCaP cells (Fig. 2A). ERG, PSA, and *TMPRSS2* (from the intact allele) mRNA were similarly induced in VCaP (Fig. 2B). Interestingly, induction was ~2-fold higher for *TMPRSS2* than *ERG*, suggesting that additional proteins may be increasing basal *TMPRSS2:ERG* expression. An AR antagonist, bicalutamide, suppressed DHT-stimulated expression of ERG (Fig. 2C). Taken together, these observations indicate that AR is similarly regulating both the wild-type *TMPRSS2* and the *TMPRSS2:ERG* fusion gene.

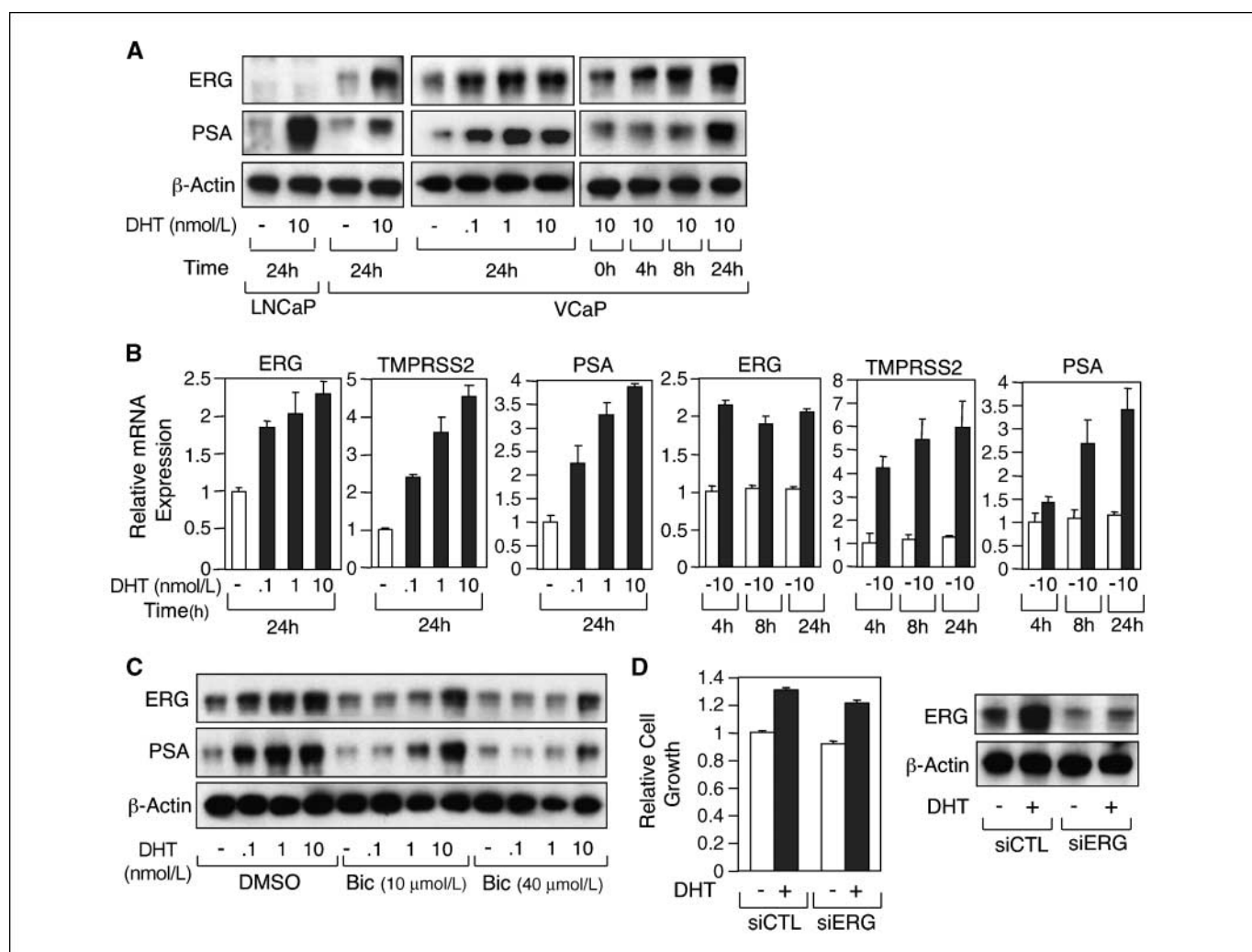


Figure 2. Androgen-regulated *TMPRSS2:ERG* expression in VCaP. **A**, cells in CSS medium were treated with DHT and immunoblotted. **B**, RT-PCR for ERG (exon 9/10), *TMPRSS2* (exon 5/6), and PSA mRNA after DHT stimulation. **C**, cells in CSS medium treated with DHT and bicalutamide (*Bic*) for 24 h. **D**, cells in 5% CSS for 3 d were transfected with 10 nmol/L control or ERG siRNA (Dharmacon). DHT was added 8 h after transfection and cells were assayed for ERG protein and cell recovery (by MTT assay) after 3 d (with comparable results at 5 d).

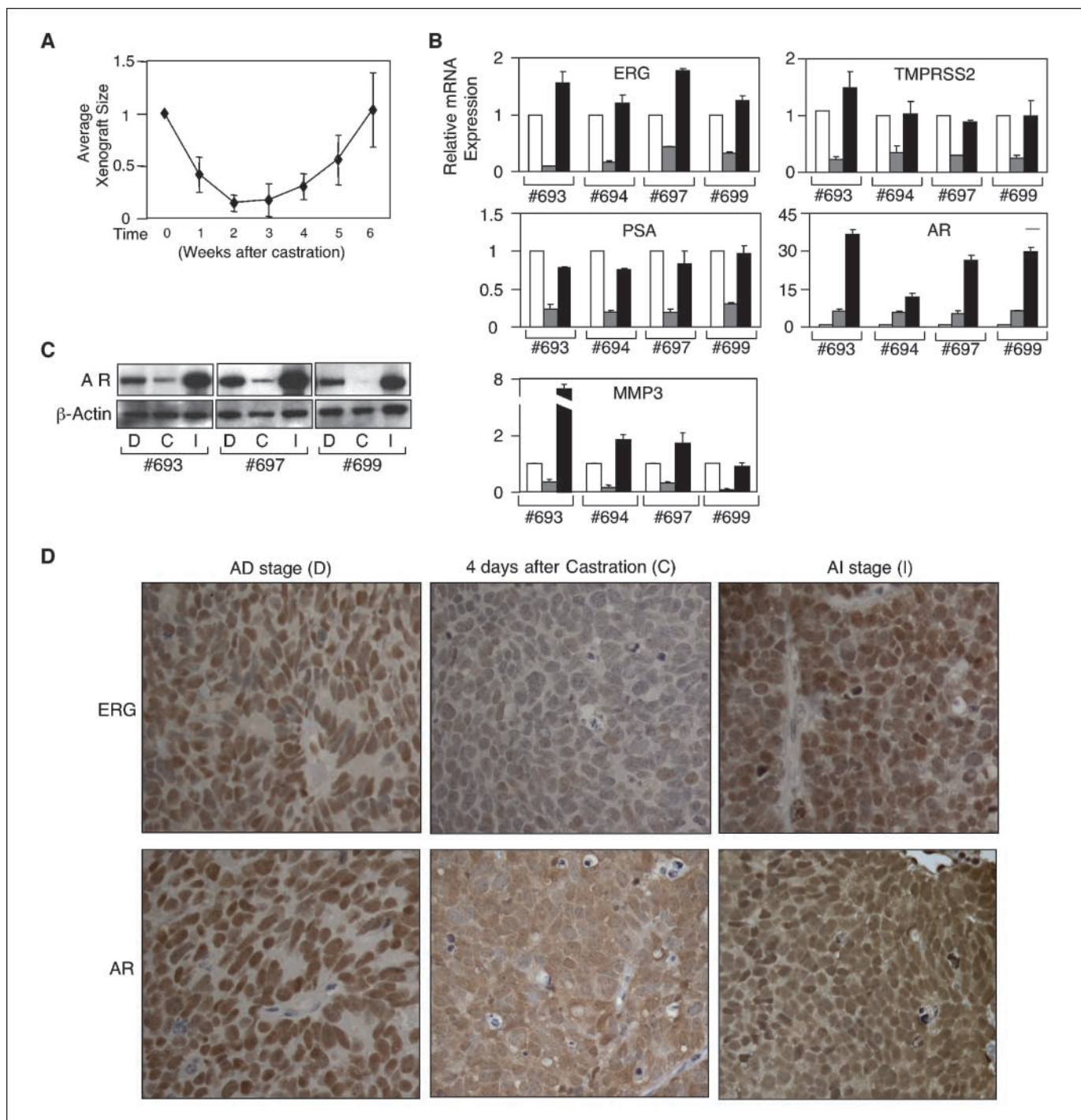


Figure 3. TMPRSS2:ERG expression in VCaP xenografts. **A**, average normalized xenograft size (\pm SD) at 1 to 6 wk after castration ($n = 6$). **B**, ERG, TMPRSS2, PSA, AR, and MMP3 mRNA in xenografts from four mice before castration (androgen-dependent, *white columns*), 4 d after castration (*gray columns*), or at relapse (androgen-independent, *black columns*). **C**, AR protein levels in xenografts before castration (*D*), 4 d after castration (*C*), and at relapse (*I*). **D**, immunohistochemistry for ERG and AR in representative xenograft.

Finally, using small interfering RNA (siRNA) to decrease ERG expression, we did not observe marked effects on cell growth in the presence or absence of DHT (Fig. 2D).

TMPRSS2:ERG expression in VCaP xenografts is ablated by castration and reactivated in relapsed tumors. S.c. VCaP xenografts were biopsied before castration, at 4 days after cas-

tration, and at ~ 6 weeks when tumors were growing rapidly and reached ~ 1 cm (Fig. 3A). As expected, PSA mRNA was decreased at 4 days and restored in the relapsed tumors (Fig. 3B). Moreover, ERG and TMPRSS2 expressions were also markedly decreased after castration and returned to precastration levels in the relapsed tumors, consistent with AR

reactivation (Fig. 3B). Although the importance of ERG expression *in vitro* remains unclear, previous data suggest that ERG functions *in vivo* by inducing genes that enhance tumor invasion, including matrix metalloproteinases (MMP) that are

established *Ets* target genes (15–17). Significantly, MMP3 expression markedly declined after castration and returned to at least precastration levels in the relapsed tumors, supporting a role for ERG in recurrence.

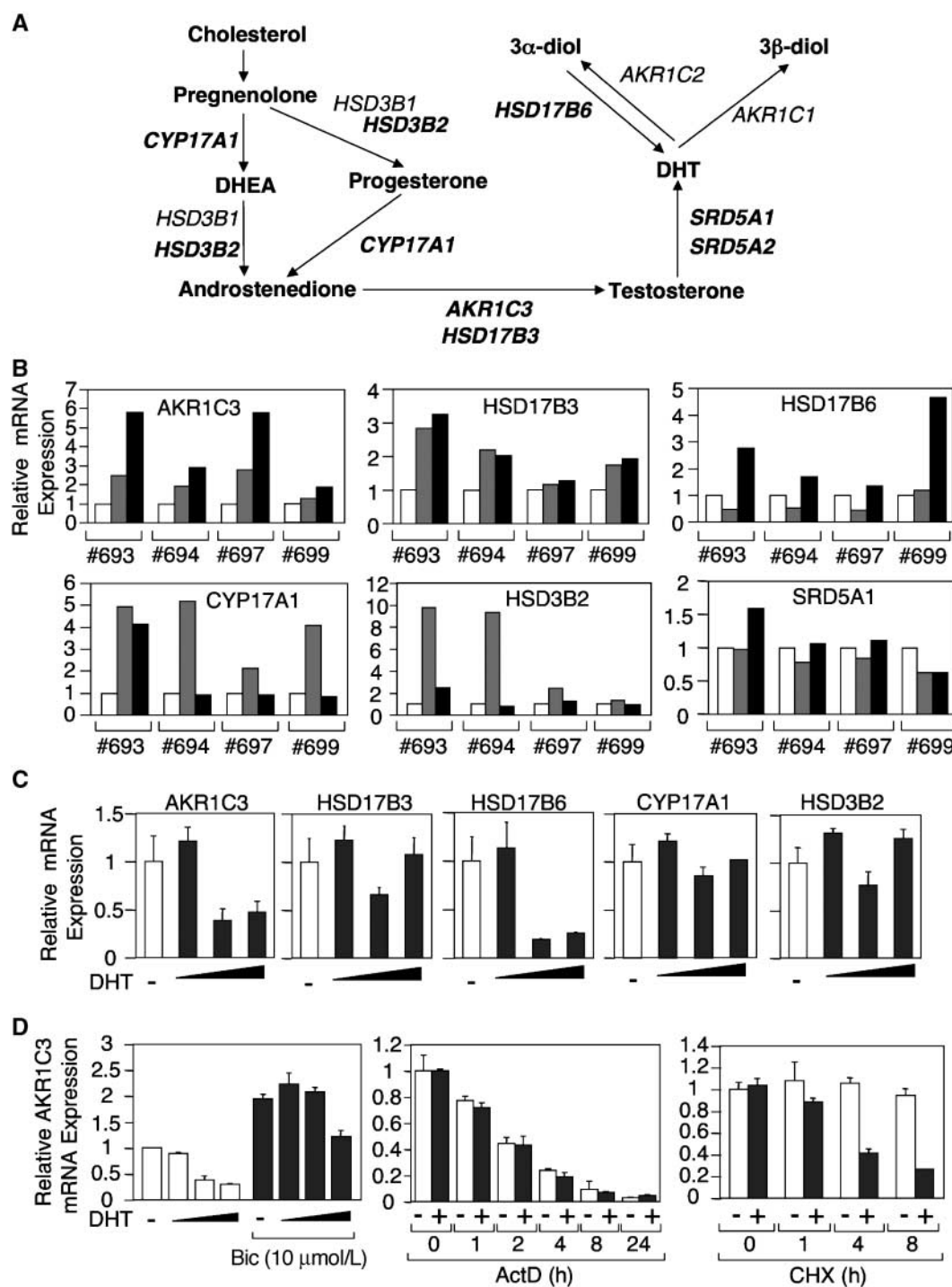


Figure 4. Androgen synthetic enzymes in VCaP xenografts. *A*, enzymes in androgen metabolism. *B*, relative expression of indicated enzymes by RT-PCR in VCaP xenografts (before castration, blank columns; 4 d after castration, gray columns; relapse, black columns). *C*, VCaP cells in CSS medium were treated with 0, 0.1, 1, or 10 nmol/L of DHT for 24 h and assessed by RT-PCR for the indicated transcripts. *D*, VCaP cells grown in CSS medium were treated with (left) 0, 0.1, 1, or 10 nmol/L of DHT minus or plus bicalutamide (10 μ mol/L) for 24 h, (middle) actinomycin D (ActD; 10 μ mol/L) minus or plus DHT (10 nmol/L) for 0 to 24 h, or (right) cycloheximide (CHX; 10 ng/mL) minus or plus DHT (10 nmol/L) for 0 to 8 h. Actinomycin D and cycloheximide were added 1 h before DHT. Results were normalized to 18S RNA.

Previous analyses of CRPC clinical samples and xenograft models have shown that AR mRNA is highly expressed and increased compared with levels before androgen deprivation (7, 8, 18, 19). Consistent with these data, AR mRNA in VCaP xenografts was increased after castration and was further increased in the relapsed tumors (Fig. 3B). In contrast, AR protein was markedly decreased at 4 days but was increased in the relapsed tumors (Fig. 3C). Nuclear ERG and AR expression were observed by immunohistochemistry in the VCaP xenografts before castration (Fig. 3D). Both declined markedly 4 days after castration, and the remaining AR at this time seemed to be cytoplasmic. Consistent with the RT-PCR and immunoblotting results, immunohistochemistry showed that both ERG and AR expressions were restored in the relapsed tumors. Whereas AR expression was primarily nuclear before castration, intense nuclear and diffuse cytoplasmic AR expressions were observed in the relapsed tumors. Taken together, these data show that *TMPRSS2:ERG* expression is markedly decreased in response to castration and that expression is restored in conjunction with AR reactivation in relapsed tumors.

Relapsed VCaP xenografts have increased expression of enzymes mediating androgen synthesis. PC may adapt to ADT by enhancing synthesis of androgens from weak steroid precursors and/or by *de novo* synthesis (8, 11, 12). Therefore, we assessed androgen synthetic enzymes before and after castration (Fig. 4A). AKR1C3 was increased after 4 days and went up further in the relapsed tumors (Fig. 4B). AKR1C3 expression in VCaP cells *in vitro* was also rapidly decreased by DHT, indicating that it is negatively regulated by androgen (Fig. 4C). Further experiments showed that this negative regulation was agonist dependent (blocked by an AR antagonist, bicalutamide) and reflected decreased transcription rather than increased mRNA degradation as it was not observed in

actinomycin D-treated cells (Fig. 4D). Finally, DHT decreased AKR1C3 mRNA in cells treated with cycloheximide, indicating that new protein synthesis was not required and suggesting that the agonist liganded AR may be directly suppressing AKR1C3 transcription.

HSD17B3 in testes carries out the same reaction as AKR1C3 but not normally in prostate. Although it was increased by castration, its basal expression was at the lower limits of detection. HSD17B6, which regenerates DHT from its major metabolite (3 α -diol; ref. 20), was also increased in the relapsed tumors (Fig. 4B) and was rapidly down-regulated by DHT *in vitro* (Fig. 4C). CYP17A1 and HSDB2 were increased after castration but returned to approximately baseline levels in the relapsed tumors (it should be noted that rodent adrenal glands do not express CYP17, so serum levels of "adrenal androgens" are low in castrate mice). These observations indicate that the entire steroidogenesis pathway is up-regulated acutely in response to ADT in VCaP xenografts and that further up-regulation of AKR1C3 and HSD17B6, in conjunction with increased AR, may eventually provide the tumors with adequate androgen for AR reactivation and expression of AR-regulated genes, including *TMPRSS2:ERG*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A phase II study of mifepristone (RU-486) in castration-resistant prostate cancer, with a correlative assessment of androgen-related hormones

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Study Type – Therapy (all or none case series)
Level of Evidence 1c

OBJECTIVE

To evaluate mifepristone (RU-486) in patients with castration-resistant prostate cancer (CRPC), with a correlative assessment of serum androgens and androgen metabolites

PATIENTS AND METHODS

The androgen receptor (AR) is critical in the development and progression of prostate cancer, but available antiandrogens incompletely abrogate AR signalling. Mifepristone is a potent AR antagonist that functions by competing with androgen, preventing AR coactivator binding and by enhancing binding of AR corepressors.

Patients with CRPC were treated with mifepristone 200 mg/day oral until disease progression. Testosterone, dihydrotestosterone (DHT), androstenedione, dihydroepiandrosterone sulphate and the testosterone metabolite 3 α -diol G, were measured at baseline and during therapy.

RESULTS

Nineteen patients were enrolled between April and August 2005; they were treated for a median (range) of 85 (31–338) days. The median prostate-specific antigen (PSA) level at enrolment was 22.0 (3.0–937.2) ng/mL. No patient had a PSA response (>50% reduction in PSA). Six patients had stable disease for a median of 5.5 months. After 1 month, adrenal androgens were increased and testosterone and DHT increased by 91% and 80%, respectively, compared to baseline.

CONCLUSION

Mifepristone had limited activity in patients with CRPC, and stimulated a marked increase in adrenal androgens, testosterone and DHT. We hypothesise that inhibition of glucocorticoid receptor by mifepristone resulted in an increase in adrenocorticotrophic hormone and subsequent increase in adrenal androgens, and that their conversion by tumour cells to testosterone and DHT probably limited the efficacy of mifepristone. These data emphasize the continued importance of alternative androgen sources in AR signalling in CRPC.

KEYWORDS

prostate cancer, secondary hormone therapy, mifepristone

INTRODUCTION

Initial androgen-deprivation therapy (ADT) of prostate cancer relies on the suppression of testicular androgen production alone or in conjunction with an androgen receptor (AR) antagonist (combined androgen blockade). While most patients respond, they eventually relapse with more aggressive tumours (termed hormone-refractory, androgen-independent or castration-resistant prostate cancer, CRPC), and effective therapy for this stage of the disease remains a serious and unmet clinical need. While CRPC develops in patients with classically defined 'castrate levels' of testosterone (<50 ng/dL), many of these patients respond to secondary

hormonal therapies that further suppress androgen levels, with a smaller fraction responding to treatment with an AR antagonist [1,2]. These responses are generally incomplete and short-lived, but they indicate that androgens and the AR still contribute to the growth of these tumours. Moreover, AR and multiple AR-regulated genes are still expressed in most CRPCs, even after secondary hormonal therapies have failed, indicating that AR transcriptional activity remains critical for CRPC growth [3–6]. The mechanisms responsible for this AR reactivation in CRPC and resistance to available hormonal therapies have not been established, but might include AR mutation or amplification, increased expression of

transcriptional coactivator proteins, or activation of signal transduction pathways that increase AR activity and render the AR 'hypersensitive' to low levels of androgens [5–8]. The activation of intracellular kinases might target the AR for phosphorylation that lowers the activation threshold to allow AR-mediated signalling in a castrate host [9,10]. A further mechanism in some CRPCs might be increased tumour cell synthesis of androgens *de novo* or from adrenal androgen precursors [3,4,11–13].

The nonsteroidal AR antagonists in clinical use (bicalutamide, hydroxyflutamide and nilutamide) are all competitive inhibitors of androgen binding to the AR. The antagonist-

ligand-bound AR either remains associated with heat-shock proteins in the cytoplasm or forms an inactive transcriptional complex on DNA, although hydroxyflutamide has some weak partial agonist activity [14,15]. AR antagonists might also enhance the recruitment of transcriptional corepressor proteins (nuclear receptor corepressor, NCoR; and silencing mediator for retinoid and thyroid hormone receptors, SMRT) that can further suppress AR transcriptional activity [16–19]. The efficacy of these AR antagonists clearly depends on their affinity for the AR relative to testosterone and dihydrotestosterone (DHT) [14]. While the binding of bicalutamide to AR is superior to hydroxyflutamide, its affinity for AR is still only $\approx 2\%$ compared to the affinity of DHT for AR, with a K_D in the micromolar range [14]. The low affinity of these antagonists compared to physiological ligands, in conjunction with adaptations that appear to enhance AR responses to low levels of androgens in CRPC, probably contribute to their limited activity in CRPC.

Mifepristone (RU486) is a progesterone analogue best known as a progesterone receptor (PR) antagonist, and was developed in France as a medical approach to terminating pregnancy [20]. Mifepristone similarly inhibits glucocorticoid receptor (GR) and has been used for treating Cushing syndrome [21]. Biochemical studies show that mifepristone binds to the hormone-binding site in the PR and GR, and functions as an antagonist due to its bulky phenyl-aminodimethyl group in the 11β position, which prevents recruitment of coactivator proteins and stimulates the recruitment of the NCoR and SMRT corepressor proteins [22]. Significantly, mifepristone is also an AR antagonist, and binding studies indicate that it has a higher affinity for AR than have hydroxyflutamide and bicalutamide [19]. Moreover, mifepristone is more effective than other AR antagonists at mediating recruitment of the NCoR and SMRT corepressor proteins [17,19].

These features of mifepristone indicated that it might be more active in CRPC than other available AR antagonists. Consistent with this hypothesis, previous studies showed that mifepristone can inhibit the growth of CRPC xenografts in castrated mice [23,24]. To test this hypothesis in patients, we conducted a phase II trial of mifepristone in CRPC. Significantly, as mifepristone is also a GR

antagonist, we included correlative hormone measurements to assess the effects of the drug on adrenal steroid production.

PATIENTS AND METHODS

For eligibility, patients required histological documentation of adenocarcinoma of the prostate, a PSA level of >5.0 ng/mL and/or bone metastasis(es) by bone scan or CT. Patients were required to have clinical, biochemical or radiographic progression after primary ADT with either orchidectomy or GnRH analogue therapy. Previous antiandrogen therapy was allowed but not required. Before entry, antiandrogen must have been discontinued for ≥ 4 weeks (flutamide) or ≥ 6 weeks (bicalutamide, nilutamide) with documentation of PSA progression (two serial rises ≥ 1 week apart) and no evidence of ongoing antiandrogen withdrawal response. GnRH analogue therapy was continued for those individuals with no orchidectomy. Previous ketoconazole, aminoglutethimide, steroids (for prostate cancer), vaccine therapy (or other immunotherapy) and oestrogens were allowed. These therapies must have been discontinued for ≥ 28 days with signs of cancer progression. No concomitant therapy with oral corticosteroids was allowed. One previous chemotherapy regimen was allowed. An Eastern Cooperative Oncology Group performance status of 0–1 was required. Patients had serum testosterone levels of ≤ 50 ng/dL.

Mifepristone at 200 mg/day was prescribed and provided to each patient. Patients kept a 'pill diary' which was reviewed by research staff. Dose modifications for toxicity were outlined in the protocol. As mifepristone is metabolized by CYP4503A4, concomitant medications were reviewed for interaction and discontinued if necessary.

Toxicity and adverse events were assessed using the National Cancer Institute Common Criteria Version 3.0. The response was assessed by measuring PSA levels every 4 weeks, and measurable disease or bone metastases were assessed every 12 weeks by CT and bone scans. A PSA response was defined as a decline from baseline by $\geq 50\%$ or normalization of PSA (defined as a PSA level of <0.2 ng/mL), confirmed by a second measurement ≥ 1 week apart. Patients could not have clinical or radiographic evidence of

disease progression during this period. The date of response was defined as the first date at which the PSA level declined from baseline by $\geq 50\%$. Stable disease (SD) was defined as patients who did not meet the criteria for response or progressive disease for ≥ 3 months. Progressive disease (PD) was determined by: (i) an increase in PSA level above baseline by $\geq 25\%$ in patients whose PSA level did not decrease; (ii) an increase in PSA level above the nadir by $\geq 25\%$ in patients whose PSA decreased but did not meet response criteria; (iii) an increase in PSA level of $\geq 50\%$ in patients who had a PSA response. In all cases an increase of ≥ 5 ng/mL confirmed by a second measurement was required to meet the definition of PD. The appearance of any new bone lesion(s) was considered progression. For target lesions on CT a complete response was the disappearance of all target lesions. A partial response was a $\geq 30\%$ decrease in the sum of the longest diameters of the target lesions; PD was a $\geq 20\%$ increase in the sum of the longest diameters, and SD was neither criteria to qualify as a partial response nor criteria for progression.

Serum was collected and stored at baseline, at 29 days and at the end of treatment. At the end of the trial the total levels of testosterone, DHT, dihydroepiandrosterone sulphate (DHEA-S), androstendione, and 5α -androstane- $3\alpha,17\beta$ -diol glucuronide (3α -diol G) in all of the samples were measured in duplicate by radioimmunoassay using kits from Diagnostic System Laboratories (Webster, TX, USA).

The Wilcoxon signed-rank test was used to test whether differences in hormone levels were different from zero (statistically significant). The Wilcoxon rank sum test was used to test for differences in hormone levels, age, PSA level at diagnosis, and number of previous hormone regimens between patients who had SD and those who did not. Fisher's exact test was used to test for differences in the proportion of patients with previous chemotherapy, previous ketoconazole, and with different proportions of total Gleason scores at diagnosis. All tests were two-sided, with $P < 0.05$ considered to indicate statistical significance.

RESULTS

Nineteen men were enrolled between April and August 2005; 18 were from the Dana-

TABLE 1 The patients' characteristics at enrolment

Characteristic	SD	Not SD	Total
Patients	6	13	19
Median (range) age, years	76 (66–79)	73 (60–85)	76 (60–85)
Gleason score, <i>n</i>			
7	2	3	5
8	0	3	3
9	3	6	9
Unknown	1	1	2
Median (range) PSA level at enrolment, mg/mL	14 (3–937)	28 (4–604)	22 (3–937)
Disease status			
Measurable	1	4	5
Bone metastases	6	12	18
Previous hormone therapy			
Orchidectomy	2	0	2
LHRH analogue	4	12	17
Oestrogen	2	2	4
Steroids	4	8	12
Ketoconazole	5	11	16
Megestrol acetate	0	1	1
Median (range):			
<i>n</i> previous hormones	6 (1–9)	6 (2–8)	6 (1–9)
Duration, months	16.2 (4–162)	6.1 (6–12)	9.1 (4–162)
Previous antiandrogen	5	13	18
Bicalutamide			
Standard dose	3	12	15
High-dose	3	3	6
Flutamide	2	1	3
Nilutamide	2	1	3
Previous chemotherapy	3	3	6
Previous ketoconazole	5	11	16

Farber Harvard Cancer Center Hospitals (Dana Farber Cancer Institute, Beth Israel Deaconess Medical Center, Massachusetts General Hospital) and one from Georgetown University. The patients' characteristics are shown in Table 1; those with SD on mifepristone and those with PD are shown separately. As a group, these patients had aggressive tumour features with a high PSA level (median baseline 22.0, range 3–937 ng/mL) and high Gleason scores (all ≥ 7 , and 71% Gleason 8/9). In addition, eight of the 19 patients had measurable disease. These patients were heavily pretreated, with a median of six previous hormonal therapies and eight having previous chemotherapy.

Patients were treated for a median (range) of 85 (31–338) days. There were no PSA responses and six patients had SD for a median of 5.5 months. The two patients who had orchidectomy had SD. Also, those with SD had previous ADT for a median (range) of

86 (16–162) months, vs 61 months for the whole group.

At the time of the present analysis, all but two patients met protocol criteria for PD. One of them had prolonged SD for >1 year; the other discontinued treatment due to toxicity after one cycle and was censored for further disease evaluation. The basis for PD was predominantly an increase in PSA level. Five patients had either bone progression documented by a scan or symptoms indicative of bone progression concurrent with PSA progression. The median (95% CI) time to progression was 1.9 (1.1–5.4) months.

The sample size was based on the primary endpoint of PSA response. A two-stage design was used to test the null hypothesis that the response rate to mifepristone would be >30%. The trial was designed to have 90% power to reject the null hypothesis when the true response rate is 50%. With a two-sided

TABLE 2 Treatment-related adverse events, as number of events

Toxicity type	Grade		
	1	2	3
Tinnitus	0	1	0
Haemoglobin	1	0	0
Neutrophils	1	0	0
Fatigue	22	1	1
Constitutional, other	2	0	0
INR	0	0	1
Rash/desquamation	1	0	0
Hot flashes	3	0	0
Anorexia	5	0	0
Dehydration	0	1	0
Nausea	11	4	0
Vomiting	0	1	0
Haemorrhage, anus	0	0	1
Haemorrhage, bladder	1	0	0
Hypercholesterolaemia	0	0	1
Metabolic, other	1	0	0
Dizziness	0	0	1
Pain, back	0	1	0
Pain, bone	1	0	0
Pain, joint	0	1	0
Pain, muscle	1	0	0
Pain, stomach	1	0	0
Pain, other	0	1	0
Dyspnoea	1	0	0
Pulmonary, other	1	0	0
Gynaecomastia	2	0	0
Worst degree	6	4	3

INR, International normalised ratio.

significance level of 10%, it was planned to enrol 22 patients in the first stage to evaluate response rate. The study was stopped after 19 patients were enrolled, as there were no responses and therefore the trial was not continued into the second stage.

Toxicities are shown in Table 2; mild fatigue and nausea were the most common. There was one episode each of grade III fatigue, increased international normalized ratio, with rectal bleeding and dizziness.

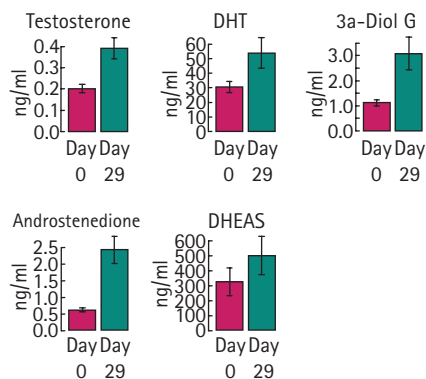
Figure 1 shows the mean (SEM) hormone levels at baseline and at 29 days; significantly, there were marked increases in serum androgen levels after 1 month of treatment with mifepristone (Table 3). In 17 patients (data were not available for two), the mean (range) baseline testosterone increased by 91 (24–224)%. The DHT levels also increased,

TABLE 3 Increases in serum hormone levels after 30 days of treatment

Hormone	Mean (SD)	Mean (SD, range) % change
Testosterone, ng/mL	0.20 (0.08)	91 (55, 24–224)
DHT, pg/mL	30.5 (16.31)	80 (93, –21 to 259)
3 α -diol G, ng/mL	1.12 (0.50)	155 (155, –44 to 502)
Androstenedione, ng/mL	0.60 (0.24)	278 (201, 9–633)
DHEAS, ng/mL	322.5 (378.64)	55 (64, –35 to 199)

Hormone	SD (six)	Not SD (11)	P	TABLE 4
Testosterone, ng/mL	0.20 (0.13)	0.19 (0.16)	0.84	The mean (SD) increase in
DHT, pg/mL	21.8 (29.5)	24.3 (34.8)	0.92	hormone levels from
3 α -diol G, ng/mL	2.58 (2.96)	1.63 (2.04)	0.31	baseline to 29 days in
Androstenedione, ng/mL	1.74 (1.70)	1.84 (1.49)	0.92	patients with SD or not
DHEAS, ng/mL	198.6 (355.8)	162.5 (356.2)	0.31	

FIG. 1. Hormone levels at baseline and at 29 days.



from a mean at baseline of 30.5 pg/mL to 54.0 pg/mL at 29 days (80% increase, $P < 0.001$). The mean androstenedione level at baseline was 0.60 ng/mL and it increased to 2.4 ng/mL after a month of therapy ($P < 0.001$), and the mean DHEA-S level increased by 55% ($P = 0.02$). At baseline the mean level of DHT metabolite 3 α -diol G was 1.12 ng/mL and it increased to 3.08 ng/mL at 29 days ($P < 0.001$). Androgen levels in patients with SD were compared to that in those with PD and the changes in hormone levels at 29 vs 1 day were not significantly different in the two groups (Table 4).

DISCUSSION

The primary goal of the present study was to assess the efficacy of mifepristone as an AR antagonist in CRPC. Available nonsteroidal AR

antagonists can effectively block AR activity in previously untreated patients with 'androgen dependent' prostate cancer, but these drugs have very limited efficacy in patients with CRPC [1,25]. The relatively low affinity of these antagonists for the AR might compromise their efficacy, although other factors might also impair their ability to block AR (see below). Preclinical data indicated that mifepristone might be more active in CRPC than these available antagonists, as it had a higher affinity for the AR and could more effectively enhance AR recruitment of corepressor proteins [17,19]. This hypothesis was tested in the current phase II study of mifepristone in patients with advanced CRPC.

Mifepristone was well tolerated, and six patients had SD; however, this is a 'soft' clinical endpoint, and all patients were progressing before starting mifepristone. We felt that 'stable' vs 'not stable' probably separated patients into biologically divergent groups. The patients with SD had a longer duration of response to primary hormone therapy than those who did not (Table 1); this characteristic has been reported in other trials of second-line hormone therapy, and the duration of response to primary androgen depletion might be a marker of hormone sensitivity in the second-line treatment setting [26]. Hormone levels (Table 4) were not significantly different in patients with SD vs other disease, but there were too few patients for definitive conclusions. There were no patients who could be classified as responders to mifepristone. The low response rate was probably influenced by these

patients having been heavily pretreated, with a mean of six previous hormone manipulations, and eight had had previous chemotherapy.

A most interesting aspect of this trial was the correlative assessment of adrenal hormone levels. These hormone measurements served as a surrogate marker to determine whether therapeutic levels of mifepristone were being achieved in these patients. Significantly, most patients had substantial increases in their levels of DHEA-S and androstenedione after 4 weeks of treatment, consistent with inhibition of the GR and a feedback increase in pituitary adrenocorticotrophic hormone production. Unfortunately, adrenocorticotrophic hormone was not a planned measurement, and as samples need to be collected on ice, it could not be measured retrospectively. Previous studies also found increases in adrenal hormone synthesis in response to mifepristone, but there are no data from castrated men [27,28]. Importantly, patients also had increases in levels of total testosterone, DHT and 3 α -diol G, reflecting peripheral conversion of androstenedione into testosterone, and subsequent metabolism to DHT and 3 α -diol G. Some increase in these androgens was clearly anticipated, as peripheral sites, including skin and normal prostate and CRPC tumour cells, can carry out these reactions. However, these are the first data to establish the magnitude of this increase in castrated men with CRPC, and to show a physiological effect of increased androgens at levels considered 'castrate'.

The substantial increases in testosterone and DHT, and the close correlation between these and the increases in adrenal androgens, clearly support the hypothesis that adrenal androgens are a significant source of testosterone and DHT in men with CRPC. It is probable that the efficacy of mifepristone was compromised by the elevated testosterone and DHT, which would be expected to strongly compete with mifepristone for AR binding.

The adrenal gland has been implicated as a source of androgens that might be stimulating cancer progression after castration for many years, and clinical responses to adrenalectomy or adrenal hormone suppression with corticosteroids, aminoglutethimide and ketoconazole have been well documented [1,29].

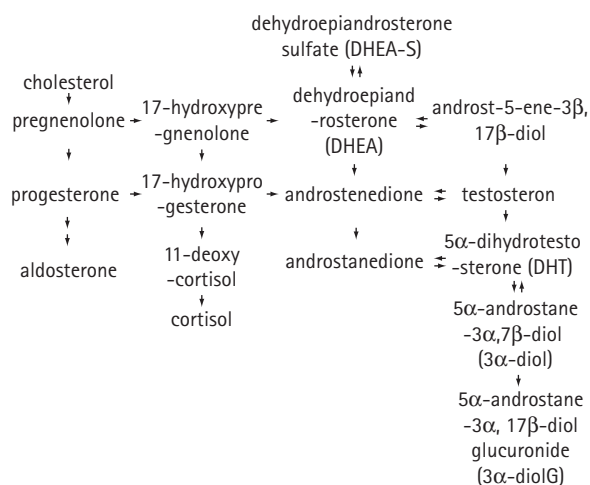


FIG. 2.
Adrenal androgen synthesis and metabolism.

Androstenedione is converted to testosterone in normal prostate and in prostate cancer cells by the enzyme aldoketoreductase 1C3 (AKR1C3) (Fig. 2) [4]. Testosterone is then reduced to the more potent DHT by 5 α -reductases (primarily type 2 5 α -reductases in normal prostate), and further enzymes then inactivate DHT and produce secreted metabolites including 3 α -diol G. Several studies indicate that this conversion of adrenal androgens to testosterone and DHT is increased in CRPC [2,4,11]. We recently showed that CRPC bone metastases had increases in many enzymes mediating DHT synthesis and metabolism from adrenal precursors, including AKR1C3 and type 1 5 α -reductase [4]. The increased expression of type 1 5 α -reductase and decreased type 2 5 α -reductase were similarly reported in other studies. Also, it was shown that CRPC specimens from local recurrences contain increased levels of testosterone and DHT relative to systemic levels, findings that support intracrine production of androgens [12,13,30].

Taken together, these data support the conclusion that CRPC is being stimulated by adrenal androgen production and subsequent conversion to testosterone and DHT, although the extent to which the increased serum testosterone and DHT are derived from tumour cells rather than other peripheral sites is not clear. The inhibition of GR by mifepristone resulted in increased conversion of adrenal androgens to testosterone and DHT, and because CRPC cells have enhanced capacity for AR activation, this increase might have abrogated any inhibitory effect of mifepristone on AR.

This is the first report of treatment with mifepristone in patients with prostate cancer. Daily dosing of mifepristone was well tolerated in this elderly population, with no incidence of clinical adrenal insufficiency. The observation that some patients had SD despite increases in serum androgens suggests that mifepristone had AR antagonist activity. The combination of mifepristone with a drug to block the compensatory rise in adrenal androgens might be effective in CRPC. Possible agents that might be used in combination with mifepristone include corticosteroids, ketoconazole or 5 α -reductase inhibitors. The development of more potent and specific AR antagonists might also lead to more effective treatment of CRPC in the future. Importantly, this work shows that the development of compounds that can more effectively suppress the androgen axis in CRPC will result in clinical benefit.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: ADT, androgen-deprivation therapy; AR, androgen receptor; CRPC, castration-resistant prostate cancer; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; DHT, dihydrotestosterone; PR, progesterone receptor; GR, glucocorticoid receptor; DHEA-S, dihydroepiandrosterone sulphate; 3 α -diol G, 5 α -androstane-3 α ,17 β -diol glucuronide; SD, stable disease; PD, progressive disease; AKR1C3, aldoketoreductase 1C3.

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Clinical Cancer Research

Phase II Study of Androgen Synthesis Inhibition with Ketoconazole, Hydrocortisone, and Dutasteride in Asymptomatic Castration-Resistant Prostate Cancer

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Phase II Study of Androgen Synthesis Inhibition with Ketoconazole, Hydrocortisone, and Dutasteride in Asymptomatic Castration-Resistant Prostate Cancer

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Abstract Purpose: Increasing evidence indicates that enhanced intratumoral androgen synthesis contributes to prostate cancer progression after androgen deprivation therapy. This phase II study was designed to assess responses to blocking multiple steps in androgen synthesis with inhibitors of CYP17A1 (ketoconazole) and type I and II 5 α -reductases (dutasteride) in patients with castration-resistant prostate cancer (CRPC).

Experimental Design: Fifty-seven men with CRPC were continued on gonadal suppression and treated with ketoconazole (400 mg thrice daily), hydrocortisone (30 mg/AM, 10 mg/PM), and dutasteride (0.5 mg/d).

Results: Prostate-specific antigen response rate ($\geq 50\%$ decline) was 56% (32 of 57; 95% confidence interval, 42.4-69.3%); the median duration of response was 20 months. In patients with measurable disease, 6 of 20 (30%) responded by the Response Evaluation Criteria in Solid Tumors. Median duration of treatment was 8 months; 9 patients remained on therapy with treatment durations censored at 18 to 32 months. Median time to progression was 14.5 months. Grade 3 toxicities occurred in 32% with only one reported grade 4 (thrombosis) toxicity. Dehydroepiandrosterone sulfate declined by 89%, androstenedione by 56%, and testosterone by 66%, and dihydrotestosterone declined to below detectable levels compared with baseline levels with testicular suppression alone. Median baseline levels and declines in dehydroepiandrosterone sulfate, androstenedione, testosterone, and dihydrotestosterone were not statistically different in the responders versus nonresponders, and hormone levels were not significantly increased from nadir levels at relapse.

Conclusion: The response proportion to ketoconazole, hydrocortisone, and dutasteride was at least comparable with previous studies of ketoconazole alone, whereas time to progression was substantially longer. Combination therapies targeting multiple steps in androgen synthesis warrant further investigation. (Clin Cancer Res 2009;15(22):7099-105)

Prostate cancer that progresses after androgen deprivation therapy (ADT), termed castration-resistant prostate cancer (CRPC), expresses androgen receptor (AR) and multiple androgen-regulated genes at high levels (including PSA and *TMPRSS2:ERG* fu-

sion genes), indicating that AR transcriptional activity has been reactivated despite castrate serum androgens levels (1-3). Mechanisms that may contribute to this AR reactivation include increased AR expression (increased AR mRNA in most patients

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Translational Relevance

Androgen receptor is reactivated in prostate cancer that relapses after androgen deprivation therapy [castration-resistant prostate cancer (CRPC)], with one mechanism being increased expression of enzymes (AKR1C3 and SRD5A1, respectively) mediating the synthesis of testosterone and dihydrotestosterone from precursor steroids. Consistent with this mechanism, inhibitors of CYP17A1 (ketoconazole or the more potent abiraterone), the enzyme that mediates androgen precursor synthesis, are active in CRPC, but responses are partial and transient. In this exploratory single-arm phase II study, we assessed the efficacy of blocking two steps in androgen synthesis by combining a dual SRD5A1/SRD5A2 inhibitor (dutasteride) with a CYP17A1 inhibitor (ketoconazole) in CRPC. The response rate and marked prolongation in time to progression compared with previous single-agent trials indicate that intratumoral conversion of low levels of testosterone to dihydrotestosterone is a mechanism of resistance to CYP17A1 inhibitors and support further combination therapy trials.

and AR gene amplification in ~30%; ref. 4), AR mutations (primarily in patients treated with an AR antagonist; refs. 5, 6), increased activity of transcriptional coactivator proteins (7, 8), and stimulation of kinases that directly or indirectly enhance AR responses to low androgen levels (9–12).

A further mechanism contributing to tumor progression after ADT is increased intratumoral androgen synthesis. CRPC tumors have increased expression of enzymes mediating testosterone and dihydrotestosterone (DHT) synthesis from weak adrenal androgens (dehydroepiandrosterone and androstenedione) and may also upregulate enzymes including CYP17A1 that are required for *de novo* steroid synthesis (3, 13, 14). Consistent with increased intratumoral androgen synthesis in CRPC, androgen levels in the prostates of men who recur locally after ADT are comparable with levels in the prostates of eugonadal men (15–17). Moreover, testosterone levels in metastatic CRPC samples are actually higher than in prostate before castration (13). Significantly, high intratumoral androgen levels, in addition to reactivating AR, may render tumor cells relatively resistant to available weak competitive AR antagonists and contribute to the modest efficacy of these antagonists when used initially in combination with castration (combined androgen blockade; ref. 18) or as secondary hormonal therapy in CRPC (19, 20).

The contribution of androgens produced by the adrenal glands to CRPC was suggested in early adrenalectomy studies (21). Ketoconazole, which inhibits several cytochrome P450 enzymes including CYP17A1 that is required for adrenal androgen synthesis, has reported response rates in CRPC ranging from 20% to 75% (22). The response rate in the largest study of ketoconazole/hydrocortisone (with simultaneous or sequential antiandrogen withdrawal) was 32% when used after antiandrogen withdrawal (23). Interestingly, this study found a positive correlation between responses and pre-therapy levels

of androstenedione, and the mean adrenal androgen levels were partially restored at disease progression (24). These observations suggest that responses might be improved with more effective androgen synthesis inhibitors, and encouraging response rates (>50%) have been reported in phase I/II clinical trials of a more potent CYP17A1 inhibitor, abiraterone (22, 25).

Although CYP17A1 inhibitors can markedly reduce levels of circulating and presumably intratumoral androgen precursors, tumor cells may still convert any available androstenedione to testosterone (mediated by the enzyme AKR1C3) and then to the higher-affinity ligand DHT (mediated by type I or II 5 α -reductase). In normal prostate, testosterone is reduced to DHT primarily by the type II 5 α -reductase (SRD5A2). Selective inhibition of type II 5 α -reductase with finasteride can decrease prostate cancer incidence, but this drug does not have clear activity in CRPC (26). Studies from several groups have shown that expression of the type I 5 α -reductase (SRD5A1) is increased in primary prostate cancer, and we found that SRD5A1 expression was further increased in CRPC (3, 27). A dual inhibitor of type I and II 5 α -reductases, dutasteride, is an effective treatment for benign prostatic hyperplasia and is under investigation for prostate cancer prevention, but its efficacy in CRPC has not been determined (28).

Based on these observations and available drugs, we hypothesized that combined inhibition of CYP17A1 with ketoconazole and inhibition of type I and II 5 α -reductases with dutasteride would be an effective therapy in CRPC. We present here results of a phase II trial of combined treatment with ketoconazole, hydrocortisone, and dutasteride (KHAD) in CRPC. The trial was designed as an exploratory study to determine whether addition of dutasteride to standard ketoconazole/hydrocortisone would improve responses to at least 50% from the 32% response in the Cancer and Leukemia Group B 9583 study (23) and would be therefore worthy of further study. Additional endpoints included time to progression (TTP) and correlations between androgen levels and responses.

Materials and Methods

Patients and eligibility. This open-label, single-arm multicenter phase II study was initiated at the Dana-Farber/Harvard Cancer Center (Beth Israel Deaconess Medical Center and Dana-Farber Cancer Institute) and conducted through the Department of Defense Prostate Cancer Clinical Trials Consortium (trial registration number NCT00673127). From June 2005 to April 2007, 57 patients were consented and started on therapy. Participating institutions included Dana-Farber/Harvard Cancer Center ($n = 26$), Sunnybrook Health Science Centre ($n = 10$), Oregon Health and Science University ($n = 8$), M. D. Anderson Cancer Center ($n = 8$), and Johns Hopkins University ($n = 5$). The institutional review board of each institution approved the trial.

Eligibility included progressive CRPC, defined as a prostate-specific antigen (PSA) increase over baseline of >25% or >5 ng/mL, or new lesions on bone/computed tomographic scan after conventional androgen deprivation and antiandrogen withdrawal. Metastatic disease was not required. Additional criteria included ongoing gonadal androgen ablation with serum testosterone <0.5 ng/mL, PSA ≥ 2 ng/mL, no prior therapy with ketoconazole or corticosteroids for prostate cancer, and Eastern Cooperative Oncology Group performance status of 0 to 2. Prior chemotherapy was allowed. Patients taking drugs that may prolong

QT intervals or known to be narrow therapeutic index CYP3A4 substrates were excluded.

The treatment was ketoconazole 400 mg orally thrice daily, hydrocortisone (30 mg/AM and 10 mg/PM), and dutasteride (0.5 mg/d). Dose modifications for toxicity were specified. Patients were evaluated every 4 weeks, with history, physical examination, and laboratory analysis including liver function tests and PSA. Serum for hormone measurements was obtained every 4 weeks for the first 12 weeks and then every 12 weeks until progression (measured in duplicate by RIA, Diagnostic Systems Laboratories). Measurable disease was evaluated by computed tomography and bone metastasis by bone scan every 12 weeks. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 3.0.

Endpoints. The primary endpoint was PSA response defined as a decline of at least 50% from baseline confirmed by a second measurement at least 4 weeks later; the reference for these declines was measured within 2 weeks before starting therapy. PSA progression was defined according to PSA Working Group Criteria (29). Measurable disease response and progression were evaluated according to the Response Evaluation Criteria in Solid Tumors. Progressive nonmeasurable disease was defined as two or more new lesions on bone scan, appearance of new nonbony metastases, or development of an indication for radiation therapy. TTP was defined from the date of treatment initiation until the date that PSA progression criteria were first met or the date of measurable or nonmeasurable disease progression; otherwise, it was censored at the date of the last PSA measurement without evidence of disease progression. Among patients who achieved a $\geq 50\%$ PSA decline, PSA response duration was defined from the date of the first 50% PSA decline until the date of PSA or disease progression or was censored at the date of the last PSA measurement without progression. Hormone levels were compared using Wilcoxon rank-sum tests.

Statistical considerations. Enrollment proceeded in a two-stage design to differentiate a response rate of $\geq 50\%$ from a response rate of $\leq 32\%$ with type I and II error of 0.10. On assessment that ≥ 8 of the initial 23 patients had responded, enrollment continued to a total of 57 patients. The PSA response rate and exact binomial 95% confidence interval (95% CI) are reported. All patients, regardless of their disease evaluations, are included in the assessment of response. The PSA response duration and TTP distributions were summarized using the Kaplan-Meier method with 95% CI.

Results

Patient characteristics. Patient and disease characteristics are in Table 1. The mean age at enrollment was 68 years, and the median duration of primary ADT was 3.4 years. Median baseline PSA was 24 ng/mL (range, 3.7-2,740 ng/mL), and median PSA doubling time before entry was 2.4 months. Measurable disease was present in 35% of patients, and 70% had bone metastases. Prior treatments included bicalutamide in 89% of patients and chemotherapy in 5% of patients.

Treatment activity. The PSA response rate (confirmed $\geq 50\%$ decline in PSA from baseline) was 56% (32 of 57; 95% CI, 42.4-69.3%). PSA declined by $\geq 80\%$ in 27 of 57 (47%) patients and by $\geq 90\%$ in 16 of 57 (28%) patients (Fig. 1). Among the 32 PSA responders, the median PSA response duration was 20 months (95% CI, 13.5 to undetermined upper limit); 9 patients were continuing on treatment without progression at the time of this report with treatment durations censored at 18 to 32 months. PSA response rates according to type of disease at baseline (measurable, nonmeasurable, PSA only) are shown in Table 2 and were similar in all groups.

Among the 20 patients with measurable disease at baseline, 6 (30%) had complete or partial responses by the Response Eval-

Table 1. Patient and disease characteristics

Characteristics	n (%) of patients or median [range] of values
No. patients	57 (100)
Non-Caucasian	5 (9)
Hispanic or Latino	1 (2)
At initial prostate cancer diagnosis	
Age (y)	61 [44-80]
PSA (ng/mL), n = 46	16 [3-1,549]
Gleason score	
4-6	14 (25)
7	16 (28)
8-9	20 (35)
Unknown	7 (14)
Prior treatments	
Duration of primary ADT (y), n = 54	3.4 [0.2-15.5]
LHRH agonist	51 (89)
Orchiectomy	4 (7)
Antiandrogens (any)	52 (91)
Bicalutamide	51 (89)
Nilutamide	6 (11)
Flutamide	5 (9)
Finasteride	2 (4)
Estrogens (including estramustine)	8 (14)
Chemotherapy	3 (5)
At registration	
Age (y)	68 [48-90]
Years since diagnosis	6.6 [0.3-20]
ECOG performance status 1	16 (28)
PSA (ng/mL)	24 [3.7-2,740]
PSA doubling time (mo), n = 51*	2.4 [1-24]
Alkaline phosphatase (units/L)	84 [30-527]
Hemoglobin (g/dL)	13.1 [10.7-15.6]
Type of disease	
Measurable disease	20 (35)
Measurable only	6 (11)
Measurable and nonmeasurable	14 (25)
Nonmeasurable disease only	28 (49)
PSA only	9 (16)
Sites of disease (multiple)	
Viscera	6 (11)
Soft tissue	6 (11)
Lymph nodes	23 (40)
Bone	40 (70)

*PSA doubling time was calculated as natural log₂ divided by the slope of the linear regression of natural log of PSA versus time using the three PSA values that confirmed progressive CRPC by PSA progression before study entry.

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

uation Criteria in Solid Tumors, 5 (25%) had stable disease, and 1 had progressive disease. The remaining 8 patients discontinued participation in the study due to PSA progression (3 patients) or for other reasons (5 patients) before measurable disease responses could be assessed. The 1 patient with a complete response had baseline PSA of 2,740 ng/mL, a 1.5 cm lymph node metastasis, and bone metastases. The target lymph node metastasis was not present on 6-, 9-, 12-, or 15-month computed tomographic scans and nadir PSA was 2.1 ng/mL at 8 months. This patient stopped treatment after ~15 months by choice with an increasing PSA of 6 ng/mL.

There were 40 patients with bone metastases at baseline. Eighteen (40%) had stable disease (0 or 1 new lesions on bone scan) throughout the therapy, and 15 of these 18 had

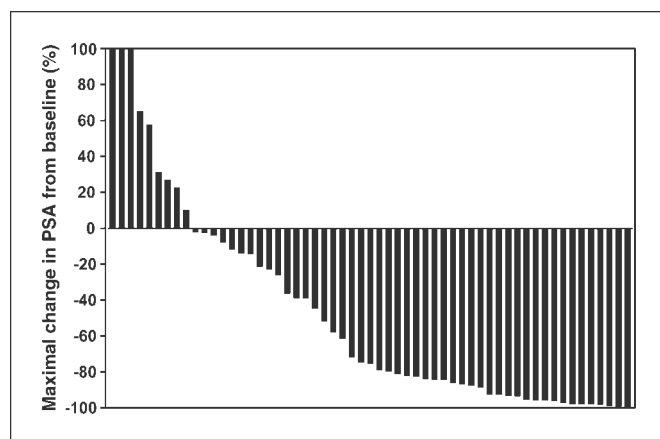


Fig. 1. Maximal change in PSA from baseline among 57 CRPC patients treated with KHAD. Y axis is truncated at +100%, but 3 patients had $\geq 100\%$ increase in PSA from baseline without decline. In total, 9 patients did not have any decline in PSA.

PSA responses. One patient had stable bone metastases for >1 year but then had bone progression resulting in treatment cessation. Eight patients had progression of bone metastases at the first bone scan (2 of these 8 patients had PSA responses within those first 3 months of treatment). The remaining 13 patients came off treatment within 3 months and did not have a follow-up bone scan (3 of these 13 had PSA progression).

The median time to disease progression was 14.5 months (95% CI, 11.0 to upper limit undetermined; Fig. 2). The median duration of treatment was 8 months, ranging from <1 to >32 months. Forty percent (22 of 57) continued treatment for ≥ 1 year. Ten patients stopped treatment with the reason specified as toxicity or side effects. An additional 10 patients stopped treatment by physician discretion or patient choice. One patient stopped treatment at 5 weeks for pain management and 1 patient stopped at 8 months for nerve root compression.

Toxicity. Toxicities were reported among all patients. Excluding impotence, 32% (18 of 57) of patients experienced at least one grade 3 or 4 toxicity. Grade 3 hypertension occurred in 4 patients, hyperglycemia in 3 patients, hypokalemia in 2 patients, and elevated alanine aminotransferase (serum glutamic oxaloacetic transaminase) in 2 patients. Grade 3 lymphopenia, rash, hot flashes, nausea, bladder hemorrhage, infection, aspartate aminotransferase (serum glutamic pyruvate transaminase), creatinine, nonneuropathic generalized weakness, cognitive disturbance, pain, dyspnea, and urinary frequency/urgency were reported each for 1 patient. There was one grade 4 thrombosis.

Hormone data. Baseline serum samples were available from 41 patients, and there was at least one subsequent on-therapy

serum sample from 33 of these 41 patients. Median dehydroepiandrosterone sulfate (DHEAS) at baseline was 599 ng/mL and fell to 65 ng/mL after ~1 month on therapy (median, 89% decline; Table 3). Baseline median androstenedione was 0.84 ng/mL and dropped by a median of 56% to 0.22 ng/mL at 1 month. Baseline median testosterone was 0.37 ng/mL, and at 1 month, there was a 66% drop to a median of 0.13 ng/mL. Baseline median DHT was 2.6 pg/mL. This declined after 1 month in all patients, with the median at 1 month being below the level of sensitivity (<2.0 pg/mL). There was no further significant decline in any of these hormones after 2 to 3 months of therapy.

Baseline median levels of androstenedione, testosterone, and DHT were similar in the patients who responded to therapy ($n = 20$) versus the nonresponders ($n = 21$; Table 4). Baseline DHEAS was higher in the responders (840 ng/mL) versus the nonresponders (480 ng/mL), but this was not statistically significant. Median decline in DHEAS after 1 month of therapy was also greater in the responders (93%) versus the nonresponders (85%), but this difference did not reach significance (Table 4). The declines in androstenedione, testosterone, and DHT were comparable in the responders versus nonresponders. Among the responding patients who had progressed at the time of this analysis, paired serum samples after 1 month on therapy and at the time of progression were available from 15 cases. There was no significant increase in the median ratio at progression versus 1 month for DHEAS (1.17; 95% CI, 0.71-1.92), androstenedione (1.11; 95% CI, 0.65-1.92), testosterone (1.34; 95% CI, 0.89-1.99), or DHT (1.11; 95% CI, 0.66-1.82).

Discussion

We conducted an exploratory phase II study to assess the efficacy of treatment with ketoconazole/hydrocortisone in combination with dutasteride in men with CRPC. The largest previous study of ketoconazole (Cancer and Leukemia Group B 9583) analyzed responses to ketoconazole/hydrocortisone given concurrently or subsequent to antiandrogen withdrawal (response rates of 27% and 32%, respectively), so the current study was powered to determine whether the response was >32% (23). Although response rates to ketoconazole in other smaller studies have ranged from 20% to 75% (22), the 56% response rate to KHAD indicates that 5 α -reductase inhibition by dutasteride may enhance the response rate to ketoconazole/hydrocortisone. In contrast to the response rate, response durations in this study were markedly longer than those reported previously for ketoconazole/hydrocortisone. The median duration of response was 20 months, and 9 of the 32 responding patients had not yet progressed at the time of this analysis (at durations of 18-32 months). Among all patients,

Table 2. PSA response rates by type of disease at baseline

	Enrolled, <i>n</i>	PSA response, <i>n</i> (%)
Measurable disease \pm nonmeasurable disease	20	11 (55)
Measurable only	6	3 (50)
Measurable and nonmeasurable	14	8 (57)
Nonmeasurable only	28	14 (50)
PSA only	9	7 (78)
Overall	57	32 (56)

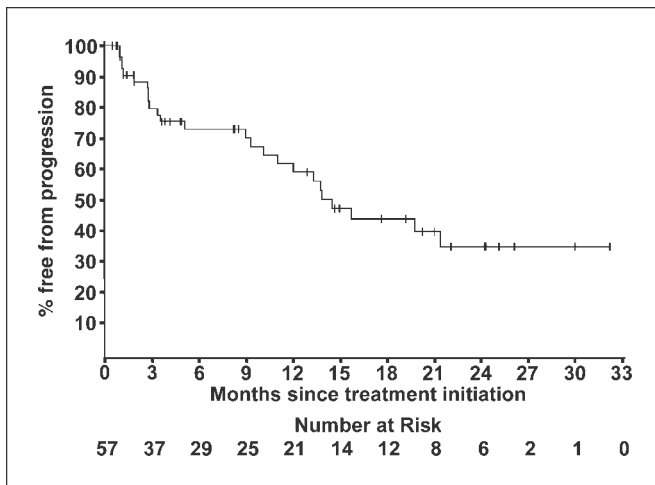


Fig. 2. TTP among 57 CRPC patients treated with KHAD.

median TTP was 14.5 months. This is markedly longer than the median time to PSA progression of 8.6 months reported in Cancer and Leukemia Group B 9583 (23) and longer than the median response durations of between 3.3 and 9 months in seven other reported ketoconazole trials (22). It is also longer than the median TTP of 7.5 months in phase II studies of abiraterone (see below; ref. 30).

As expected, KHAD caused a median decline of ~90% in serum DHEAS levels and declines of ~50% to 70% in serum androstenedione and testosterone. Baseline median levels of androstenedione and testosterone were comparable among responders and nonresponders, whereas median DHEAS was higher in the responders (840 versus 480 ng/mL, respectively), although this difference was not statistically significant. In contrast, higher baseline androstenedione and DHEAS levels were correlated with responses to ketoconazole/hydrocortisone plus antiandrogen withdrawal in the Cancer and Leukemia Group B 9583 study, although only the androstenedione association was significant. A further finding in Cancer and Leukemia Group B 9583 was that median levels of DHEAS and androstenedione, which had declined after 1 month of therapy, were increased relative to nadir

levels at the time of progression. We did not observe a consistent increase in any of the measured hormones at relapse, but samples from patients who are still responding remain to be examined.

DHT also declined in all patients, but basal levels were already at the limit of sensitivity and the median level after 1 month was below the sensitivity of the assay. Previous studies have not examined the effects of ketoconazole/hydrocortisone alone on DHT in castrate men, so we cannot determine the extent to which DHT levels were further suppressed by dutasteride or assess correlations between declines in serum DHT and responses. Moreover, concentrations of DHT (and of testosterone) in the tumor are likely higher than serum levels and may more closely correlate with responses (13, 16, 17).

Abiraterone is a more potent and specific CYP17A1 inhibitor than ketoconazole, with phase I studies showing >95% declines in serum testosterone to <0.01 ng/mL (25). Nonetheless, despite this more marked decrease in serum testosterone levels, the reported response rate to abiraterone in phase I/II studies (>50% PSA decline in 67% and >90% PSA decline in 19%) appears comparable with the KHAD response rate (25, 30). Moreover, as noted above, median time to PSA progression on abiraterone was 7.5 versus 14.5 months for KHAD. These observations suggest that intratumoral conversion of weak androgens to testosterone and DHT may still be contributing to treatment resistance and relapse in abiraterone-treated patients. In contrast, the addition of dutasteride to ketoconazole, by blocking intratumoral conversion of testosterone to DHT, may result in extremely low intratumoral DHT levels that compensate for the somewhat higher levels of residual testosterone. It also should be noted that dutasteride will presumably increase the aromatization of testosterone to estradiol and decrease the levels of DHT metabolites that may be estrogen receptor- β ligands, although the net effect of this altered testosterone metabolism on tumor growth is uncertain. In any case, the hypothesis that intratumoral DHT synthesis contributes to abiraterone resistance should be tested in a randomized phase II trial.

Early studies employed adrenalectomy or hypophysectomy as therapies for CRPC, with about one-third of patients having objective responses in measurable disease and the majority having subjective improvement (21). Adrenalectomy clearly ablates adrenal androgen synthesis, but there are likely other sources

Table 3. Hormone levels over time among all patients

Hormone	Baseline value	Month 1		Month 2		Month 3	
		Value	Percentage change from baseline	Value	Percentage change from baseline	Value	Percentage change from baseline
Patients, <i>n</i>	41	33	30	28	24	28	24
Androstenedione (ng/mL)	0.84 (0.61, 1.10)	0.22 (0.20, 0.36)	-56 (-75, -43)	0.27 (0.20, 0.32)	-68 (-74, -49)	0.25 (0.20, 0.31)	-58 (-77, -49)
DHEAS (ng/mL)	599 (340, 1206)	65 (36, 125)	-89 (-94, -70)	65 (37, 106)	-92 (-94, -77)	65 (35, 100)	-90 (-94, -81)
Testosterone (ng/mL)	0.37 (0.26, 0.47)	0.13 (0.10, 0.15)	-66 (-72, -55)	0.11 (0.9, 0.14)	-68 (-73, -54)	0.12 (0.10, 0.19)	-64 (-72, -45)
DHT* (pg/mL)	2.6 (<2.0, 3.8)	<2.0 (<2.0, <2.0)					

NOTE: Median and interquartile range (25th, 75th percentiles).

*For DHT, *n* = 33 at baseline, *n* = 32 at month 1, and *n* = 29 for percent change.

Table 4. Hormone levels at baseline and percentage change at month 1 according to PSA response at 3 months

Hormone	Baseline			Percentage change from baseline to month 1		
	PSA responders	PSA nonresponders	P*	PSA responders	PSA nonresponders	P*
Patients, n	20	21		14	16	
Androstenedione (ng/mL)	0.82 (0.6, 1.18)	0.85 (0.63, 0.98)	0.98	-58 (-73, -43)	-56 (-77, -45)	0.82
DHEAS (ng/mL)	840 (397, 1,145)	480 (311, 1,491)	0.56	-93 (-95, -85)	-85 (-91, -61)	0.1
Testosterone (ng/mL)	0.33 (0.26, 0.50)	0.39 (0.23, 0.47)	0.91	-66 (-74, -56)	-61 (-70, -49)	0.29
DHT [†] (pg/mL)	2.3 (<2.0, 3.7)	3 (<2.0, 4.0)	0.63			

NOTE: Median and interquartile range (25th, 75th percentiles).

*P value by Wilcoxon rank-sum test comparing PSA responders versus PSA nonresponders.

[†]For DHT, at baseline, n = 16 and 17 for PSA responders and nonresponders, respectively, and n = 16 and 15 for percent change from baseline to month 1, respectively.

of weak androgens, including CRPC cells that may express increased levels of CYP17A1, although it remains to be established whether CRPC cells synthesize substantial levels of androgens *de novo* from cholesterol (13, 14). In any case, we suggest that adrenalectomy does not reflect maximal androgen deprivation and that androgen levels (particularly intratumoral levels) may be further reduced by more potent inhibitors of androgen synthesis or by combinations of inhibitors that block at multiple steps.

In summary, this study indicates that dutasteride may enhance the response rate to ketoconazole/hydrocortisone and substantially increases the duration of response and overall TTP. This result supports the conclusion that DHT synthesis by the type I 5 α -reductase, which is increased in CRPC, contributes to prostate cancer survival and progression after ADT. Further randomized trials of 5 α -reductase inhibitors in conjunction with ketoconazole or more potent CYP17A1 inhibitors are warranted. Moreover, further efforts should be made to develop inhibitors of other enzymes mediating androgen synthesis. Finally, it will clearly be important to determine whether these

efforts to more effectively suppress androgen synthesis and AR activity lead to improved survival.

Note Added in Proof

Plasma dutasteride concentrations measured in 6 patients by a validated LC/MS-MS method ranged from 80–140 ng/ml approximately 2–3 times higher than the mean 40 ng/ml levels in patients on dutasteride monotherapy at the same dose. This finding is in agreement with the Dutasteride product label describing the potential for CYP3A4 inhibitors such as ketoconazole to impair dutasteride metabolism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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